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THE TYPHUS GROUP OF DISEASES IN MALAYA.—PART I: THE STUDY OF THE VIRUS OF RURAL TYPHUS IN LABORATORY ANIMALS. PART II: THE STUDY OF THE VIRUS OF TSUTSUGAMUSHI DISEASE IN LABORATORY ANIMALS.

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GENERAL INTRODUCTION.

The typhus-like diseases of Malaya are members of that large and ever-enlarging group of typhus-like fevers, one or more of which may exist within the confines of one country, and even of circumscribed areas of comparatively small extent. Thus, in the Federated Malay States, within a radius of twenty miles from Kuala Lumpur, three members of the group have frequently been encountered, namely, the rural type of tropical typhus, the urban type of tropical typhus and the tsutsugamushi disease.

In recent years successive discoveries of new endemic foci in the Old and New Worlds and in the Far East have added to the complexity of the typhus group, and given an impetus to research in this field, especially to that directed towards the elucidation of the degree of relationship between the various entities of the group.

During the past four year the authors have established in laboratory animals strains of the three typhus diseases of Malaya from human sources, have studied their characteristics, and the relationship of the three viruses with one another and with that of Rocky Mountain spotted fever; strains of typhus have been isolated from wild rats; the problem of transmission in nature has been investigated and, in part, elucidated. Preliminary reports have been published in successive annual reports from this Institute (1931, 1932, 1933 and 1934), and a résumé of one phase of the work read at the Ninth Congress, Far Eastern Association of Tropical Medicine (1934).

It is proposed to present the report of the above investigations as a consecutive series of papers in this Journal.

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PART I: THE STUDY OF THE VIRUS OF THE RURAL TYPHUS* IN LABORATORY ANIMALS.

Rural typhus is an acute infectious disease that is characterized by onset of fever and severe headache; by a rash that appears about the fifth day; by nervous manifestations; and by termination by crisis or lysis about the end of the second week. Clinically it closely resembles typhus exanthematicus. In epidemiology, however, it differs entirely, for it does not flare up in devastating epidemics, nor is it louse-borne. To mark this similarity and contrast with the typhus of the western world, Fletcher and Lesslar (1925), who first recognized the disease to exist in Malaya in 1924, designated it as "tropical" typhus. In the following year these workers (1926) recognized a second type of tropical typhus to exist in Malaya, differing from the first type in serology and epidemiology. Insomuch as the incidence of the first type was exclusively amongst those who lived, worked or sought recreation in the countryside, while that of the second type was predominantly a disease of the house and the town, they called the first type the "rural" form of tropical typhus, the latter the "urban" form of tropical typhus.

The description of the clinical features of rural typhus, and successive reports of investigations into its serology, ætiology and epidemiology, will be found in previous publications from this Institute (see References, p. 20).

EXPERIMENTAL RURAL TYPHUS IN GUINEA-PIGS.

Previous Investigations.

The earliest attempts to infect guinea-pigs were those of Fletcher and Lesslar (1925). The disease being at that time but recently recognized, and the cases few and sporadic, opportunity for laboratory investigation occurred only late in the course of the disease, not earlier than the 12th day of fever. Of 6 cases in which guinea-pig inoculation was made, 4 gave a negative result in the first generation; in the other 2 a febrile reaction could not be reproduced beyond the second generation of guinea-pigs, and Fletcher and Lesslar regarded their results as inconclusive.

One of us (R. L.) has reported (1930) a larger series, in which 62 guinea-pig were inoculated, usually intra-peritoneally, with blood drawn from 43 patients at an earlier stage of the disease (4th–8th day); of these animals 3 only gave a febrile reaction. Guinea-pigs were inoculated also with brain, spleen and kidney emulsions from fatal human cases; none reacted. Attempts to propagate strains from the 3 reacting guinea-pigs failed at the 5th generation.

Anigstein (1933) reported the inoculation of 200 guinea-pigs with infected human blood or brain, and a further 240 with virus from passage animals and other sources; no more than 11 p.c. gave a febrile reaction, and in one instance only could a strain be maintained as far as the 12th generation. Attempts

* The "rural" form of tropical typhus is also variously referred to in the literature as "scrub-typhus" and as "K-typhus"; similarly, the "urban" form of tropical typhus is frequently referred to as "shop-typhus" and as "W-typhus". The first of the three designations will be adhered to in each case in this series of papers, and for brevity will be written as "rural typhus" and "urban typhus" respectively.

by him to infect guinea-pigs selected from a group known to be susceptible to typhus exanthematicus proved equally unsuccessful.

The Isolation and Maintenance of a Strain of Rural Typhus in Guinea-pigs.

At the end of 1931 the authors, after failure to infect guinea-pigs by intra-testicular inoculation and successive passage by this route, renewed the attempt to infect the guinea-pig with virus of human origin by using only vitamin-deficient guinea-pigs. For some days before and after inoculation they were fed on a diet consisting of water, autoclaved rolled oats, and skimmed milk, this being the diet used successfully by Zinsser, Castaneda and Seastone (1931) to increase the virulence of infection with the endemic typhus of the United States of America—based on the historic association of famine with enhancement of virulence in epidemics of typhus exanthematicus.

Numerous attempts were made by us; all failed but one. In the one exception 2 guinea-pigs that had been fed on a vitamin-deficient diet for 10 days were inoculated intra-peritoneally with blood from a patient (Seerangayee), who showed the clinical syndrome of rural typhus, and who came from an estate on which this disease has been endemic for many years. The Weil-Felix reaction proved subsequently to be positive to high titres. Both guinea-pigs reacted with fever, and from these a strain has been maintained by continuous passage, and is now in its 97th generation in the guinea-pig. The characteristics of this "Seerangayee" strain of rural typhus are as follows:

The incubation period following intra-peritoneal injection of virus varies between 5 and 18 days; in the majority its limits are 7 to 12 days. Fever follows, rising during 3 days from a normal morning temperature of 102° F., to reach a maximum of 104° F.–105° F. In those guinea-pigs that survive defervescence begins about the 5th to the 6th day, and is completed usually about the 9th day. Many variations of temperature curve are seen; the fever may range between 103° F. and 104° F. only; it may be as short as 7 days or as long as 15 days (Charts I and II). No swelling of the scrotum has been observed in any of the 300 or more male guinea-pigs that have been infected with this virus. The mortality amongst those guinea-pigs inoculated by the intra-peritoneal route is fully 90 p.c., death occurring on the 4th to 5th day, and being preceded by a sudden fall of the temperature to subnormal limits. Those that survive show extreme weakness.

The two cardinal post-mortem signs are enlargement of the spleen and the presence of ascites. The splenic enlargement is always striking after the second day; the spleen is increased in size three to four times, and its surface coarsened in most cases, in contrast to the smoothness of the surface of the spleen in guinea-pigs infected with urban typhus. The ascites develops rapidly from the 3rd day of fever onwards; the fluid is slightly viscid and fibrinous. In an animal that has been infected by the intra-peritoneal route it is invariably present, and usually abundant; if the route has been subcutaneous it is usually absent or scanty. If the guinea-pig dies or is killed late in the fever, a layer of fibrin is commonly present on the surface of the spleen, and appears to be a deposit from the ascitic fluid. *Rickettsia* are always present in the

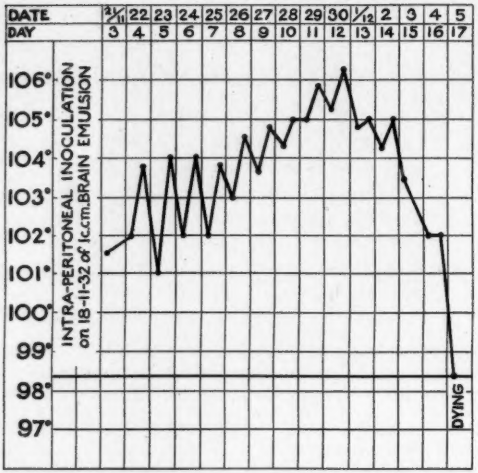

CHART I: RURAL TYPHUS.—Guinea-pig 1076. A passage animal of the "Seerangayee" strain of rural typhus. Inoculated by the intra-peritoneal route.

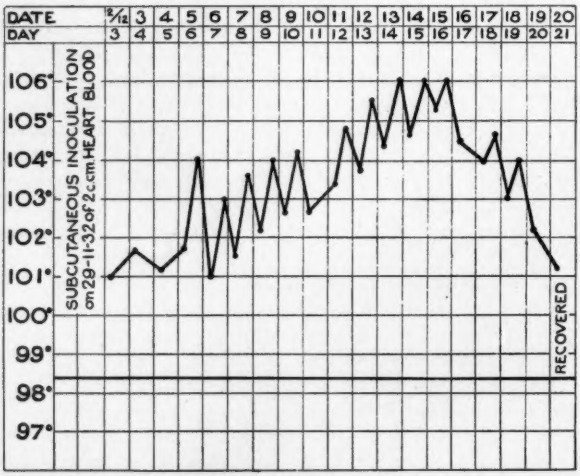

CHART II: RURAL TYPHUS.—Guinea-pig 1095. A passage animal of the "Seerangayee" strain of rural typhus. Inoculated by the subcutaneous route. As compared with the infection in guinea-pig 1076, note the longer incubation period, the more gradual evolution of fever, and recovery of the animal, three features that are characteristic of infection by the subcutaneous route.

ascitic fluid in numbers that increase with each succeeding day of fever; in the fibrinous deposit on the spleen they are copious. Less frequent post-mortem signs are injection of the suprarenal glands, enlargement and injection of the glands of the groin and axilla, and punctiform subpleural hæmorrhages. "Typhus nodes" are to be found on histological examination of the brain, but are not a conspicuous feature. Perivascular collections of mononuclear cells are, however, a prominent feature in the mesentery, being readily demonstrable in fragments of this tissue that have been spread on to slides and stained by Giemsa's method.

The severity of the experimental infection is diminished considerably by employing the subcutaneous route of inoculation; not only is the ascites less, or absent, but survival is the rule. The subcutaneous route of inoculation serves also to minimize troublesome secondary infection. As inoculum, heart-blood, ascitic fluid, and emulsions of brain, spleen or suprarenals are effective. Failure of a guinea-pig to react is extremely rare when brain-virus is injected by the intra-peritoneal route; heart-blood is less efficacious than brain, the subcutaneous route less certain to secure infection than is the intra-peritoneal route.

The brain retains the virus for some few days after defervescence; no special investigation to determine the number of days has been made, the point having merely been noted following use of the brain of a convalescent guinea-pig as an inoculum in an emergency. A series of about 30 infected brains, some in pure glycerine, others in 50 p.c. glycerine, others dry, were frozen in ice, or kept at a temperature of 0°–5° C. for periods of time varying from 2 to 30 days, with a view to determining the length of time during which the virus might remain virulent at these temperatures. Whereas control inoculations of the fresh brain were all positive, inoculation of the preserved brains showed that the virulence for the guinea-pig had been entirely lost. Bruynoghe and Jadin (1933) report much the same degree of failure in the case of material from guinea-pigs infected with a strain of "typhus murin", being unable to preserve the virulence of brain-virus with certainty at 4° C. for more than two days. The method of preservation recommended by Laigret and Durand (1933), namely, to freeze material to a temperature of −10° to −12° C., has not been tried.

After convalescence guinea-pigs are immune for a period of at least 15 months.

Although, as has been stated above, failure of a passage animal to react is now very rare, this was far from being the case at the outset. During the first 9 or 10 generations the maintenance of the strain was always precarious—much more so than was the case in the initiation of a strain of urban typhus (as will be reported later). After that period, however, infection became more and more constant, and eventually the use of vitamin-deficient animals could be discontinued.

It was noted during periods of apparent diminution of virulence that resort to the inoculation of mixed emulsions of brains from 2 or more feebly-reacting guinea-pigs restored the febrile reaction to its usual intensity.

During the maintenance of this and other typhus strains in guinea-pigs, intercurrent infections have been a constant source of anxiety, especially

when the guinea-pigs were fed on a diet deficient in vitamin A. Three contaminants that have been encountered, *B. enteritidis* Gaertner, *Spirillum morsus muris* and a *Toxoplasma*, simulate typhus in the guinea-pig both in febrile reaction and post-mortem appearances, and may supplant the typhus virus. *B. paratyphosus B* and *B. whitmori* have also been isolated, the latter giving a scrotal reaction similar to that met with in some forms of experimental typhus in the guinea-pig. It is proposed to amplify our experiences of these contaminating infections later in this series of papers.

The Demonstration of Rickettsia.

Anigstein (1933) has reported the finding of *Rickettsia* in smears from the tunica vaginalis of a guinea-pig that reacted to inoculation with the virus of rural typhus.

In the passage animals of the " Seerangayee " strain *Rickettsia* are constantly to be found in the shed endothelial cells that lie in the ascitic fluid, increasing in number as the infection progresses. They are most abundant in the fibrin on the splenic surface (Figs. 1, 5 and 6) ; cultures of this fibrin on ordinary laboratory media are sterile in the large majority of cases. In morphology, staining reactions and distribution these *Rickettsia* do not differ in any way from those demonstrated by the authors (1934) in the endothelial cells lining Descemet's membrane of rabbits infected by the intra-ocular route (also *vide infra*). Stained by Giemsa's method the organisms appear as short rod-shaped bipolar-staining bacilli. With optimum differentiation they are seen to consist of two polar granules that are stained purple and are connected by a pale blue rod. Their most characteristic grouping is that of one or more clusters that lie in greatest concentration close to the nucleus ; but occasionally they lie scattered beyond the ruptured cell margin, as though prolific multiplication within the cytoplasm has caused rupture of the cell. Average limits of dimensions may be stated as 0·8–2·0μ in length, and 0·3–0·5μ in width. Some variation in length and width is seen, but the essential structure is constant. They appear to be identical with the *Rickettsia orientalis* described by Nagayo and his collaborators (1931), and regarded by them as the causal organism of the tsutsugamushi disease, specimens of which were very kindly furnished to the authors for comparative study by Prof. Nagayo. They appear also to be identical with the diplococcal forms described by Anigstein (1933). [To facilitate appreciation of the dimensions of the *Rickettsia*, a photomicrograph of a smear of pneumococci in the blood of a mouse, taken at a magnification of 1700, is included (Fig. 10).]

EXPERIMENTAL RURAL TYPHUS IN RABBITS.

Previous Investigations.

It has previously been shown by Anigstein (1933) and by one of us (Lewthwaite, 1930) that the intra-peritoneal inoculation of the virus of rural typhus into rabbits provoked in the sera agglutinins (" O " type) to the " K " strain of *Proteus X.19* ; agglutination has, however, been low in titre and fitful in

incidence. Thus Anigstein reported maximum titres of 1 : 125 to 1 : 250, and drew attention to the vagaries of the development of agglutinins, especially to the influence of the idiosyncrasies of individual rabbits in this respect, and to the influence of the origin and quantity of the inoculated virus, as stressed by Felix (1933). In the cases previously reported by one of us, 11 rabbits were inoculated intra-peritoneally with blood drawn from patients at the height of fever ; in 8 the Weil-Felix reaction was negative ; in the other 3 the titres were positive, but did not exceed 1 : 125. No fever occurred. Later, an attempt was made to initiate strains in rabbits by intra-testicular inoculation of human blood drawn early in the disease. No fever, orchitis or positive Weil-Felix reaction developed.

It was clear, then, that the rabbit was far from being an ideal animal in which to study the characteristics of the virus of rural typhus.

The work of Nagayo and his collaborators (1931), however, on the infection of the rabbit with the virus of the kindred tsutsugamushi disease by the intra-ocular route offered the prospect of much more promising results, insomuch as inoculation had resulted in the evolution of an intense iridocyclitis, easily observed in all its stages, that ultimately disappeared to leave the eye normal, the animal healthy and immune. Moreover, the causal organism of the tsutsugamushi disease was found in abundance in material from the eye. If it were found that the virus of rural typhus evoked a similar reaction, then a new criterion of infection would be available, and one that would enable cross-immunity experiments to be made with ease.

The Isolation and Maintenance of Strains of Rural Typhus in Rabbits by the Intra-ocular Inoculation of the Virus.

The method used was that practised by Nagayo and his collaborators, viz. the inoculation of virus into the anterior chamber of the eye of the rabbit. The virus used for initiation of the strain was defibrinated blood, drawn from the patient as early as possible in the course of the disease.

From 12 patients seen early in the disease it has been possible to initiate 4 strains ; a 5th strain has been initiated from the above-mentioned " Seerangayee " guinea-pig strain of rural typhus by inoculation of blood from the heart of a reacting guinea-pig. Maintenance of these 5 strains by intra-ocular inoculation of infected aqueous humour afforded no difficulty. They were carried on for 45, 29, 17, 7 and 4 generations respectively, *i. e.* sufficiently long to permit cross-immunity and other experiments to be made ; they were then discontinued because of the excessive drain on the available supply of rabbits. Our experience has been that in attempting to isolate a strain, at least 2, preferably 3 or 4, rabbits should be used, for success with a single rabbit is uncertain. Similarly, to secure reaction in the second and third generations, it may be necessary to pass the aqueous humour from a feebly reacting rabbit of the previous generation on more than one occasion into more than one rabbit, according as the intensity of the ocular signs dictates. Once a strain has survived the first three passages, failure of a rabbit to react to inoculation of passage virus is extremely rare—less than 1 p.c.

Technique.—The details of technique given below do not differ in essentials from those set forth by Nagayo and his co-workers, on which they have been modelled. The rabbit is anæsthetized by ether, an eye speculum inserted to retract the eyelids, and the eye washed successively with solutions of 1 p.c. phenol and boric acid. The fine needle of a 1 c.c. syringe is inserted into the anterior chamber, as great a part of its course as possible being directed intracorneally in order to minimize efflux of the inoculated fluid when the needle is ultimately withdrawn. To steady the eyeball counter-pressure is applied by a pair of forceps from the opposite side. Approximately 0·1 c.c of the normal aqueous humour is withdrawn, the barrel of the syringe is then detached from the needle, is replaced by the barrel of another syringe containing 0·1 c.c. of blood-virus or infected aqueous humour ; this fluid is inoculated and the syringe with its needle withdrawn. Infected aqueous humour, when required as an inoculum, is obtained from a reacting rabbit by a similar technique. One eye only is inoculated ; the other serves as a control.

The ocular reaction.—The evolution of the reaction, which is an acute iridocyclitis, is as follows :

(*a*) An incubation period of 4–15 days, being fairly constant at 4–7 days after the first few generations. Within 24 hours of the inoculation of infected blood, during the incubation period of the true reaction there occurs a non-specific reaction—a degree of circumcorneal injection and injection of the iris—which subsides in 3 to 4 days. Where the inoculum is passage virus, *i. e.* infected aqueous humour, this immediate reaction is very slight or even absent, and does not exceed 48 hours in duration.

(*b*) The appearance of circumcorneal injection, and, one day (or two) later, of iris injection and turbidity of the aqueous humour ; these signs progress for 6 or 7 days to a marked degree of intensity, and then the appearance of pannus marks the beginning of retrogression ; the turbidity of the aqueous humour is the last sign to disappear. The duration of active signs is 12–16 days usually, though occasionally longer.

In reacting rabbits the aqueous humour is infective from the first appearance of ocular signs until at least 2 days after pannus has become evident. The maximum infectivity is present from the 4th to 6th day of iris injection and turbidity of the aqueous humour. The evolution of signs is constant, and has been identical in all the 5 strains of rural typhus. It corresponds in all respects to that described by Nagayo and his collaborators in the case of the tsutsugamushi disease.

The infected rabbits show no sign of generalized ill-effects, and all recover. Febrile reaction is fitful or absent entirely. The infected aqueous humour is almost invariably sterile on ordinary laboratory media ; in the very rare instances in which secondary infection has appeared, it has disappeared in the passage animals of the next one or two generations. After convalescence the rabbits are immune to reinfection for a period of at least 12 months. (A detailed report of a large series of cross-immunity experiments will appear in a later paper.)

Demonstration of the virus.—The procedure of Nagayo was again followed. From time to time infected rabbits were killed at the fastigium of the ocular reaction, the cornea of the reacting eye was excised, and scrapings made of the endothelium covering Descemet's membrane. Stained by Giemsa's method these scrapings reveal within the endothelial cells clusters of short rod-shaped bipolar-staining bacilli, that in morphology, staining characteristics and distribution cannot be distinguished from the *Rickettsia* noted above as being present in infected material from the " Seerangayee " guinea-pig

strain of rural typhus, from those present in reacting eyes of rabbits infected in this laboratory with strains of the tsutsugamushi disease (*vide infra*), and from the *Rickettsia orientalis* of Nagayo (Figs. 2, 3 and 7). Whereas in some scrapings many cells containing *Rickettsia* are found in every microscopic field, in others long search may be required before they are found; but they are invariably to be found.

The Weil-Felix Reaction in Rural Typhus.

It is proposed to devote a separate report in this series to the Weil-Felix reaction in rural typhus, urban typhus and the tsutsugamushi disease, and detailed discussion is therefore deferred. The findings in rabbits inoculated with human and passage virus of rural typhus are here given in brief. In all, sera were submitted for the Weil-Felix reaction from 55 inoculated rabbits. Twenty-nine of these had been inoculated by the intra-peritoneal route; most of them with brain or other tissue virus from the "Seerangayee" strain of rural typhus, in order to confirm, by the result of the Weil-Felix reaction, that the signs of reaction in passage guinea-pigs were still those of typhus, and were not due to one or other of the contaminants that in the guinea-pig may simulate typhus and supplant the typhus virus. Of these 29, in 18 the sera showed significant agglutination, maximum titres lying between limits of 1/50–1/200 in 16 and 1/200–1/300 in 2. The remaining 26 rabbits had been inoculated by the intra-ocular route, and of these, 12 showed significant agglutination, maximum titres lying between 1/50–1/200 in 9, 1/200–1/300 in 1, and 1/300–1/400 in 2. Agglutination was of the "OXK" strain of *Proteus X.19*, never of the "Warsaw" strain (the "OX19" strain in use in this laboratory).

The presence of significant agglutination was judged not merely by the titres attained, but also by the form of the curves of these agglutination titres, which must show the waxing and waning features to which Fletcher and Lesslar (1926) draw attention as constituting conclusive proof of active infection with the virus of tropical typhus.

Infection of Rabbits by the Intradermal Inoculation of Virus.

As part of a series of experiments designed to elucidate the non-occurrence of an initial ulcer in rural typhus in man, and its occurrence in the tsutsugamushi disease in man, rabbits and monkeys were inoculated intradermally with virus from strains of rural typhus. Monkeys proved to be the more susceptible, so that detail of the method is deferred until the reaction in monkeys is described. The following is a summary of the results obtained in rabbits: Six rabbits were inoculated on separate occasions with the virus of rural typhus, 5 of them with infected peritoneal fluid from a reacting guinea-pig, 1 with material from an infected eye of a reacting rabbit. Macules, rapidly progressing to papules, developed at the sites of inoculation in all 6; in 4 central necrosis ensued, to give the characteristic appearance of the primary eschar

of the tsutsugamushi disease in man (*vide* Fig. 11). Control reactions carried out with saline and with the aqueous humour of normal rabbits yielded no trace of skin reaction.

The Weil-Felix reactions showed a slight rise of agglutination titres of the " OXK " strain in sera from 2 of the 6 rabbits, but not to a conclusive degree.

EXPERIMENTAL RURAL TYPHUS IN THE WHITE RAT.

During the past seven years numerous attempts have been made in this laboratory to infect wild (brown) rats with the virus of rural typhus, attempts prompted by the discouraging outcome of attempted infection of guinea-pigs. The results have proved unsatisfactory, the incidence of fever being fitful, and, when obtained, of no more than 2 or 3 days' duration. Moreover, recent work by Anigstein (1933) has shown that in a considerable proportion of wild rats trapped in the countryside, the serum yields a positive Weil-Felix reaction ; while the authors (1931) have isolated in guinea-pigs two strains of tropical typhus from wild rats trapped in areas in which cases of human tropical typhus had occurred. Use of wild rats for isolation or maintenance of strains of rural typhus is therefore fraught with difficulties.

Nicolle (1933) recently drew attention to a distinction between the virus of Old World typhus (*le typhus historique*) and the virus of rat typhus (*le typhus murin*) that is founded on their differing behaviour to attempted propagation in the white rat ; whereas the former could not be maintained by him beyond the 13th generation in three separate series of white rats, the latter can be maintained experimentally in the white rat indefinitely.

An attempt was made to classify the " Seerangayee " strain of rural typhus by this experimental criterion. Hitherto white rats had not been used in experimental typhus work in this laboratory, since they had proved difficult to rear in the damp heat of this country. But a batch obtained in 1933 from India throve sufficiently to promise an adequate supply for the experiment, and two parallel series of strains were initiated in white rats by intra-peritoneal inoculation of brain virus from a reacting passage guinea-pig of the rural typhus strain. As in the brown rat, the incidence of febrile reaction proved fitful, and the duration of any such reaction was 2 to 3 days only. No scrotal swelling occurred. The optimum time for passage in each individual case was determined by the febrile reaction of a control guinea-pig.

At the 7th generation the white rat of one series died overnight ; and, on account of considerable decomposition, it was deemed advisable to continue the two parallel series by inoculation of virus from the rat of the remaining series into two fresh rats. Both series were maintained successfully until the 11th generation, when the control guinea-pig of one series was febrile for one day only. Lest this should have been a rare example of insusceptibility of an individual guinea-pig, passage of virus from the corresponding rat was continued. Again, the control guinea-pig showed only a short febrile reaction. In two further passages the control guinea-pig entirely failed to react, so that, in this series, the maintenance of the virus failed at the 13th generation. The other

of the two parallel series, however, was maintained successfully, with no evidence of any abatement in virulence, for 21 generations ; at this point the investigation was arbitrarily terminated for reasons of economy.

Rabbits that were inoculated intra-peritoneally with virus from white rats or control guinea-pigs at various stages during the maintenance of this strain in the white rat have, in many instances, yielded significant agglutination of the " OXK " strains of *Proteus X.19*. Further, smears from the tunica vaginalis of male white rats have revealed, usually after long search, characteristic examples of intra-cellular *Rickettsia* indistinguishable from those found in material from infected guinea-pigs of the rural typhus strain. At post-mortem examination of infected rats the only constant finding was a marked enlargement of the spleen ; but frequently ascites and a fibrinous deposit on the surface of the spleen were noted, smears of the latter yielding *Rickettsia*. Of those rats not killed to provide emulsions for passage, the majority died late in the second week or early in the third week.

It having proved possible to maintain a strain of rural typhus in white rats for 21 generations with unabated virulence, there would seem to be no reason to doubt that propagation of the strain in the rat could have proceeded indefinitely ; and that, therefore, by Nicolle's criterion, rural typhus is a murine strain. In another investigation, to be described in a later contribution in this series, the authors have isolated from wild (brown) rats two strains of rural typhus ; serologically they did not differ from strains isolated from human cases ; cross-immunity experiments were carried out with one of them, and showed this rat strain and the human strains to be immunologically identical. This evidence points strongly to the rat as being the reservoir of rural typhus, as maintained by Fletcher and his co-workers, and by Anigstein. The above successful propagation in white rats of the laboratory strain of rural typhus not only affords additional evidence as to the rodent reservoir, but also accords with the conclusion of Nicolle that typhus virus of rat origin can be maintained indefinitely in rats, in the laboratory as in nature.

A further point of interest emerging from the propagation of this rural typhus strain in white rats was the failure to evoke a scrotal swelling in any of those 32 control guinea-pigs that were males. Pinkerton (1931) noted a periodicity in the occurrence of scrotal swelling in guinea-pigs in the case of the " Wolbach " strain of Old World typhus. Further, he found that he could cause re-appearance of the scrotal swelling during a period of its non-occurrence by interpolating the inoculation of a white rat in his series of passages, and continuing the strain in guinea-pigs by intra-peritoneal inoculation of spleen and testicular emulsions from this rat.

It was hoped to find a similar phenomenon in the case of the rural typhus strain, which, unlike the urban strain to be described later, has never yet afforded an example of scrotal swelling ; and so not only to gain this additional valuable diagnostic sign of infection with the virus, but also to furnish a rich supply of *Rickettsia* that might serve as a basis for attempted production of a prophylactic vaccine against rural typhus. But although inoculation of guinea-pigs with emulsions of spleen and/or testicular washings of the rats constantly provoked the febrile reaction, in none of the 32 males was any degree of scrotal reaction evident.

EXPERIMENTAL RURAL TYPHUS IN MONKEYS.

The authors (1934) have summarized the many common features shared by rural typhus and the tsutsugamushi disease ; in particular, their lethality, epidemiology, serology, behaviour of the respective viruses in the guinea-pig and the rabbit, and the apparent identity of the respective *Rickettsia*. The clinical syndrome of the two infections in man is closely similar ; but, as noted earlier, there exists one outstanding distinction, clear-cut in all but one of a large number of cases seen by the authors, that serves as a differential criterion, namely, the presence in the tsutsugamushi disease and the absence in rural typhus of a small round ulcer, characteristic in its black necrotic centre and surrounding red areola, and an attendant painful bubo of the nearest group of lymphatic glands.

To furnish experimental data regarding this distinction, intradermal inoculation of monkeys and rabbits with the respective viruses was practised. Comparison of the results obtained is deferred to a later paper ; it is proposed here to report only the features of experimental rural typhus in monkeys.

One gibbon and 3 macacus monkeys were inoculated on different occasions. To provide inoculum for the gibbon and 1 macacus monkey, a rabbit strain of rural typhus was established by the intra-ocular inoculation of infected heart-blood from the " Seerangayee " guinea-pig strain ; and aqueous humour and scrapings of the endothelium lining Descemet's membrane were secured at the height of the ocular reaction from passage rabbits. As inoculum for the two remaining macacus monkeys, peritoneal fluid and fibrin from the surface of the spleen were obtained from a reacting guinea-pig of the " Seerangayee " rural typhus strain. Seven to 9 intradermal injections, spaced along either side of the mid-line of the abdomen, were made in each case. Cultures of the various inocula were sterile on ordinary laboratory media in every case.

In all 4 animals active infection followed. Following an incubation period of 5 days in 3 of the animals and 10 days in the other, a febrile reaction occurred that persisted for 9 days in the case of the gibbon and for 4, 14 and 2 days respectively in the case of the 3 macacus monkeys (*vide* Charts III and IV).

At every site of inoculation in all 4 monkeys lesions developed that passed through a macular to a papular stage. In each monkey some of the papules progressed further to end as small circumscribed ulcers, with the black necrotic centre and surrounding hyperæmic areola that are typical of the initial lesion of the tsutsugamushi disease in man, this final necrotic stage being secured with more certainty by inoculation of teased endothelium covering Descemet's membrane rather than aqueous humour alone ; by inoculation of fibrin rather than peritoneal fluid alone (Figs. 12 and 13). The macular stage, usually only faintly discerned, appeared one day prior to the papular stage, which appeared variously from the 3rd to the 7th day after inoculation. The black necrotic centre, where present, was clearly evident 3 to 7 days after the first appearance of the papule.

Swelling of the lymphatic glands of the groin was seen in 3 of the 4 cases. In the gibbon it was especially prominent as a large, raised, tender bubo, of the size of a walnut, that appeared on the 8th day, reached its maximum on the 10th day, to recede on the 11th day (Fig. 12).

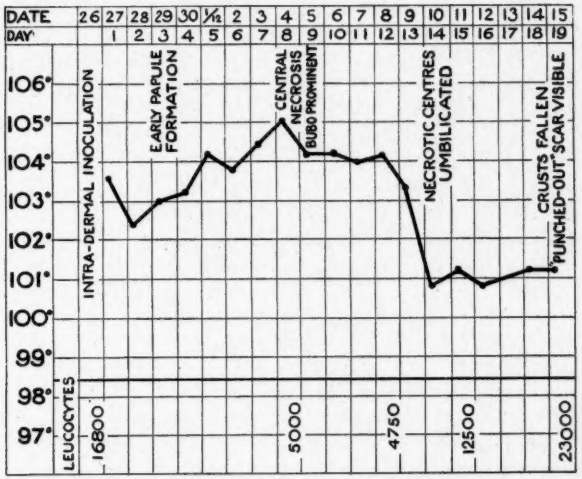
DATE 26 27 28 29 30 1/12 2 3 4 5 6 7 8 9 10 11 12 13 14 15
DAY 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
106° 105° 104° 103° 102° 101° 100° 99° 98° 97°
INTRA-DERMAL INOCULATION
EARLY PAPULE FORMATION
CENTRAL NECROSIS
BUBO PROMINENT
NECROTIC CENTRES UMBILICATED
CRUSTS FALLEN
"PUNCHED-OUT" SCAR VISIBLE
LEUCOCYTES
16800
5000
4750
12500
23000

CHART III: RURAL TYPHUS.—Gibbon No. 2. Inoculated intradermally on November 26th, 1934, with infected material from the eye of a passage rabbit of a strain of rural typhus. For dermal lesions, see Fig. 12.

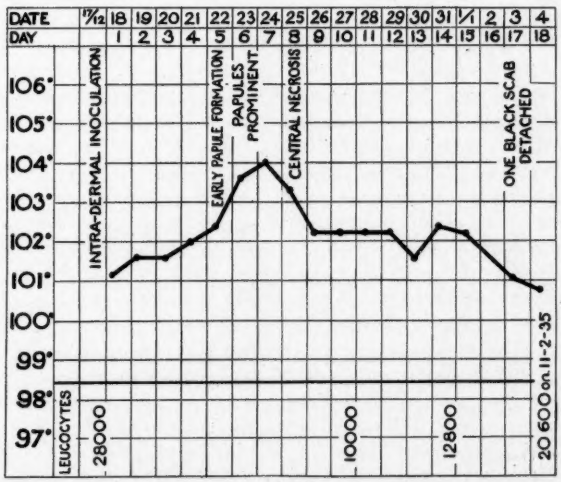

CHART IV: RURAL TYPHUS.—Monkey No. 4 (a macacus.) Inoculated intradermally on December 17th, 1934; source of inoculum, and strain, as for gibbon No. 2. For dermal lesions, see Fig. 13.

EXPERIMENTAL RURAL TYPHUS IN MONKEYS.

The authors (1934) have summarized the many common features shared by rural typhus and the tsutsugamushi disease ; in particular, their lethality, epidemiology, serology, behaviour of the respective viruses in the guinea-pig and the rabbit, and the apparent identity of the respective *Rickettsia*. The clinical syndrome of the two infections in man is closely similar ; but, as noted earlier, there exists one outstanding distinction, clear-cut in all but one of a large number of cases seen by the authors, that serves as a differential criterion, namely, the presence in the tsutsugamushi disease and the absence in rural typhus of a small round ulcer, characteristic in its black necrotic centre and surrounding red areola, and an attendant painful bubo of the nearest group of lymphatic glands.

To furnish experimental data regarding this distinction, intradermal inoculation of monkeys and rabbits with the respective viruses was practised. Comparison of the results obtained is deferred to a later paper ; it is proposed here to report only the features of experimental rural typhus in monkeys.

One gibbon and 3 macacus monkeys were inoculated on different occasions. To provide inoculum for the gibbon and 1 macacus monkey, a rabbit strain of rural typhus was established by the intra-ocular inoculation of infected heart-blood from the " Seerangayee " guinea-pig strain ; and aqueous humour and scrapings of the endothelium lining Descemet's membrane were secured at the height of the ocular reaction from passage rabbits. As inoculum for the two remaining macacus monkeys, peritoneal fluid and fibrin from the surface of the spleen were obtained from a reacting guinea-pig of the " Seerangayee " rural typhus strain. Seven to 9 intradermal injections, spaced along either side of the mid-line of the abdomen, were made in each case. Cultures of the various inocula were sterile on ordinary laboratory media in every case.

In all 4 animals active infection followed. Following an incubation period of 5 days in 3 of the animals and 10 days in the other, a febrile reaction occurred that persisted for 9 days in the case of the gibbon and for 4, 14 and 2 days respectively in the case of the 3 macacus monkeys (*vide* Charts III and IV).

At every site of inoculation in all 4 monkeys lesions developed that passed through a macular to a papular stage. In each monkey some of the papules progressed further to end as small circumscribed ulcers, with the black necrotic centre and surrounding hyperæmic areola that are typical of the initial lesion of the tsutsugamushi disease in man, this final necrotic stage being secured with more certainty by inoculation of teased endothelium covering Descemet's membrane rather than aqueous humour alone ; by inoculation of fibrin rather than peritoneal fluid alone (Figs. 12 and 13). The macular stage, usually only faintly discerned, appeared one day prior to the papular stage, which appeared variously from the 3rd to the 7th day after inoculation. The black necrotic centre, where present, was clearly evident 3 to 7 days after the first appearance of the papule.

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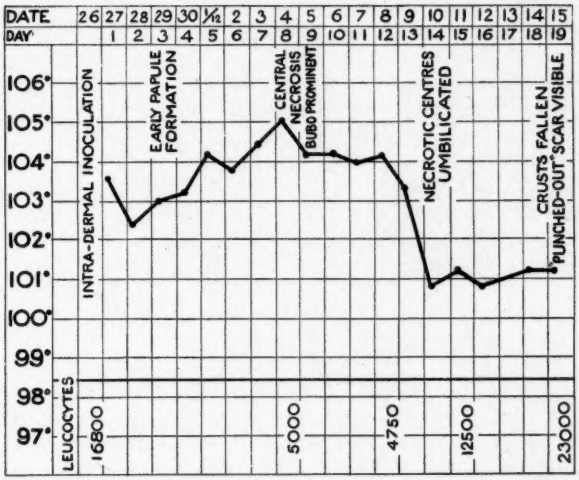

CHART III: RURAL TYPHUS.—Gibbon No. 2. Inoculated intradermally on November 26th, 1934, with infected material from the eye of a passage rabbit of a strain of rural typhus. For dermal lesions, see Fig. 12.

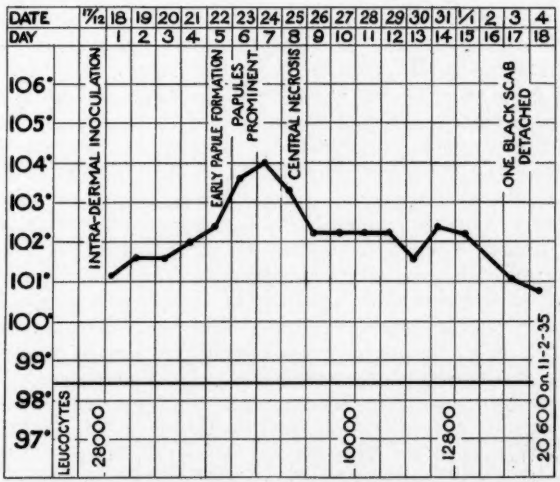

CHART IV: RURAL TYPHUS.—Monkey No. 4 (a macacus.) Inoculated intradermally on December 17th, 1934; source of inoculum, and strain, as for gibbon No. 2. For dermal lesions, see Fig. 13.

Leucopenia, characteristic in rural typhus and the tsutsugamushi disease in both man and monkey, was a well-defined feature in all 4 of these monkeys infected with rural typhus, as the following table indicates:

	(0).	(8).	(12).	(15).	(19).
Gibbon . .	16,800 .	5000 .	4750 .	12,500 .	23,000
	(0).	(10).	(15).	(56).	
First macacus .	28,000 .	10,000 .	12,800 .	20,500	
	(0).	(10).	(19).	(24).	
Second macacus .	22,500 .	16,400 .	15,000 .	20,000	
	(0).	(9).	(16).		
Third macacus .	12,500 .	8750 .	17,000		

(Figures in brackets = number of days after inoculation.)

The three macacus monkeys suffered only a mild illness, and survived the infection. The gibbon was more seriously affected; during the febrile reaction it crouched in the corner of its cage, inactive and disinclined to eat. For some days after defervescence it improved, but it died from a cellulitis, that accompanied a flaring-up of an old skin disease, on the 13th day after defervescence and 26th day after inoculation (*vide* Chart III).

In all 4 monkeys the Weil-Felix reaction was carried out with 5 to 10 successive samples of blood, drawn from the day of inoculation onwards until late in convalescence. In all 4 the reaction was positive, agglutination reaching a significant degree, and showing the waxing and waning titres indicative of active infection. In the case of the 3 macacus monkeys the maximum titres were, respectively, 1/141 on the 14th day, 1/125 on the 19th day, and 1/125 on the 28th day after inoculation; agglutination was of the "OXK" strain of *Proteus X.19*, never of the "W" (= "Warsaw") strain (the "OX19" strain in use in this laboratory). In the gibbon, agglutination of the "OXK" strain was also obtained to a maximum titre of 1/170 on the 21st day, but a predominant "O" type agglutination of the "W" strain also occurred in a maximum dilution of 1/1925 on the 15th day. In view of the interest of this dual agglutination, a strain that had been established in rabbits by intra-ocular inoculation of heart-blood from this gibbon was maintained through several generations. The rabbit of the first generation yielded a characteristic ocular reaction, and a positive Weil-Felix reaction of the "W" type only; it proved immune to reinoculation with virus of the parent "Seerangayee" (*i. e.* "K") strain of rural typhus—a result that is in entire contrast to the complete and invariable lack of cross-immunity between the viruses of rural (*i. e.* "K") and urban (*i. e.* "W") typhus, as will be shown later. Converse cross-immunity experiments between parent and daughter strain also showed complete cross-immunity to exist. Subsequent rabbits inoculated with the daughter strain failed to give a Weil-Felix reaction of the "W" type, but gave ocular reactions typical of infection with virus of rural strains, and, in one instance, a positive Weil-Felix reaction of the "K" type. Thus, in this instance, the passage of virus of a rural typhus strain from the guinea-pig, through the rabbit, to the

gibbon, caused a temporary change in its serological properties, but no change in its immunological character. Later, to afford further data, a second gibbon was inoculated intradermally ; this gibbon had three months previously reacted to intradermal inoculation of the virus of the tsutsugamushi disease with febrile reaction, leucopenia, dermal lesions, an agglutination of the " OXK " strain of *Proteus X.19*, to a titre of 1/3850, and absence of any trace of agglutination of the " W " strain. At the time of reinoculation the " K " agglutinins had fallen to zero. Peritoneal fluid from a reacting guinea-pig of the parent " Seerangayee " rural typhus strain was inoculated at multiple sites into this second gibbon, and into a macacus monkey as a control. In the gibbon, leucopenia alone resulted ; no fever or dermal lesions occurred ; there was no rise of agglutinins of the " K " strain ; and no trace of agglutination of the " W " strain, such as would have been expected to develop had the " Seerangayee " rural typhus strain contained any " W "-type virus. The control macacus monkey reacted with fever, dermal necrotic ulcers, leucopenia, and significant agglutination of the " K " strain of *Proteus X.19*, but no trace of agglutination of the " W " strain.

This rare appearance of agglutination of the " W " strain by the sera of animals infected with a virus that almost invariably provokes agglutination of the " K " strains only, with or without concomitant agglutination of the " K " strains of *Proteus X.19*, has been encountered more than once by the authors in the course of experimentation in this laboratory (I.M.R. Annual Report, 1932). A similar unexpected result is recorded by Anigstein (1933). Several human cases of tropical typhus that have shown high agglutination (" O "-type) of both " K " and " W " strains have been noted. Experiments to elucidate this paradox are in progress, and will be reported later in this series of papers, in the discussion on the inter-relationship of the Malayan typhus fevers.

PART II : THE STUDY OF THE VIRUS OF THE TSUTSUGAMUSHI DISEASE IN LABORATORY ANIMALS.

Previous reports from this Institute have dealt fully with the clinical and epidemiological characteristics of the tsutsugamushi disease in man, as encountered in Malaya, and have stressed their close similarity with those of rural typhus. This similarity amounts almost to identity, save for the occurrence of the primary ulcer and bubo in the tsutsugamushi disease. Until, in a later paper in this series, the results of cross-immunity experiments have been reported, it would be premature to discuss differential criteria ; and this present account will be restricted to the behaviour of the virus in the guinea-pig, rabbit and monkey.

ATTEMPTS TO INFECT GUINEA-PIGS WITH THE VIRUS OF THE TSUTSUGAMUSHI DISEASE.

Attempts to infect guinea-pigs by the intra-peritoneal inoculation of infected human blood have been few in number through lack of opportunity, the incidence of the disease as compared with that of rural typhus being infrequent. Fletcher and Field (1927) report three such attempts ; the authors have made

eight, the blood being taken between the 5th and 10th days of fever. In all, continued maintenance of a strain has proved impossible, in so far as febrile reaction is regarded as a criterion of successful infection. Usage of the vitamin-deficient diet, described earlier in connection with rural typhus, has not availed to overcome the marked degree of insusceptibility of the guinea-pig. Occasionally an early halting success, amounting to a precarious maintenance of a strain for a few generations, would be obtained; but invariably failure ensued. The following is a record of one such experience:

From a patient showing the typical clinical syndrome of the disease, and whose serum subsequently developed high agglutination of the "OXK" strain of *Proteus X. 19*, blood was drawn on the 7th day of fever, and inoculated into 2 guinea-pigs by the intra-peritoneal route and into 2 rabbits by the intra-ocular route. Whereas in the rabbits a strain was isolated and maintained without difficulty, in the guinea-pigs success was short-lived. Precariously, and by extravagant use of guinea-pigs, the strain was maintained for 9 generations. In the 9th generation 6 out of 8 animals failed to react; in the 10th generation none reacted. Those guinea-pigs of the earlier generations that reacted with fever after an incubation period of 8 to 13 days showed the characteristic features of the "Seerangayee" strain of rural typhus, viz. absence of scrotal swelling; the presence of subpleural hæmorrhages, ascites, and enlargement of the spleen occasionally; and, less often, a deposit of fibrin on the surface of the spleen, smears of which, when stained by Giemsa's method, revealed abundant *Rickettsia* indistinguishable in morphology, distribution and staining characteristics from *Rickettsia orientalis*.

THE ISOLATION AND MAINTENANCE OF STRAINS OF THE TSUTSUGAMUSHI DISEASE IN RABBITS BY THE INTRA-OCULAR INOCULATION OF VIRUS.

The authors have attempted thus to isolate strains of this disease from 5 patients seen by them before defervescence. Two of the 5 were not seen until the second week of fever, at a period in the evolution of the disease at which the prospect of success was remote; and the attempts failed. The other 3 patients came under observation during the first week of fever, at which period the prospect of success could be regarded as favourable, and from 2 of them strains were isolated.

Blood was drawn from the first patient on the 4th day of his fever, and inoculated into one rabbit by the intra-peritoneal route, and into 4 rabbits by the intra-ocular route. The rabbit inoculated intra-peritoneally gave a positive Weil-Felix reaction, its serum agglutinating the "OXK" strain of *Proteus X.19* only. Of the 4 rabbits inoculated intra-ocularly, 2 failed to develop either ocular signs or agglutinins in the serum to strains of *Proteus X.19*; the third developed agglutinins in the serum to the "OXK" strain, and a mild ocular reaction that could not be reproduced on passage; the fourth developed similar agglutinins, and also the ocular signs described by Nagayo and his collaborators as characteristic of the tsutsugamushi disease in rabbits thus inoculated.

Blood was drawn from the second patient on the 7th day of his illness and inoculated into 2 rabbits by the intra-ocular route. Both developed agglutinins to the "OXK" strain of *Proteus X.19*; a characteristic ocular reaction developed in one, but failed to develop in the other.

By intra-ocular passage of infected aqueous humour, precisely as described

above in the case of strains of rural typhus, these two strains of the tsutsugamushi disease were maintained without difficulty. One strain, called the "Raub" strain, has been maintained as a stock laboratory strain since its isolation in June, 1932, and is now in its 99th generation in the rabbit. The other strain, called the "Kepong" strain, was maintained sufficiently long (9 generations) to allow cross-immunity experiments to be completed, and was then discarded.

The evolution of the ocular reaction in the case of the tsutsugamushi disease is identical in every feature with that described above in the case of strains of rural typhus, and need not be re-described. As also noted above, the infected rabbits survive the infection, showing no generalized ill-effects; febrile reaction is insignificant or absent. After convalescence, the rabbits are immune to reinfection for a period of at least 12 months.

Again the virus may be demonstrated to be rickettsial in nature precisely as described in the case of rural typhus; and in abundance, distribution, morphology and staining characteristics the *Rickettsia* cannot be distinguished from the *Rickettsia* of rural typhus and from the *Rickettsia orientalis* of Nagayo (Figs. 4, 8 and 9).

The Weil-Felix reactions are also similar, significant agglutination being of the "OXK" strains, occasionally to a very high titre; the "W" strains are never agglutinated. The following examples are given here as representative of the maximum degree of agglutination that has been attained:

Tsutsugamushi strains: Rabbit No. 670—1/385 on 34th day.
,, ,, 918—1/440 on 60th day.
,, ,, 1012—1/500 on 10th and 15th day.
,, ,, 1041—1/1925 on 42nd day.

INFECTION OF RABBITS BY THE INTRADERMAL INOCULATION OF THE VIRUS OF THE TSUTSUGAMUSHI DISEASE.

Complementary to corresponding inoculations of the virus of rural typhus (*vide supra*), rabbits and monkeys were inoculated intradermally with the virus of the tsutsugamushi disease.

Three rabbits were inoculated, the inoculum consisting of infected aqueous humour and scrapings of the endothelium lining Descemet's membrane, secured at the height of the ocular reaction from reacting rabbits of the "Raub" strain. One rabbit, inoculated with infected aqueous humour only, failed to react. Each of the other 2 rabbits was inoculated with both aqueous humour and scrapings of endothelium. In one of these papules developed on the 4th day at all four sites of inoculation, but did not progress to central necrosis; agglutination of the "OXK" strain of *Proteus X.19* also developed to a significant titre. In the other of the two, macules developed on the 2nd to 5th days at four of five sites of inoculation, progressed to form papules on the 3rd to 5th days, and underwent central necrosis on the 5th to 6th days; the rabbit died of intercurrent infection on the 11th day, so that development of a positive Weil-Felix reaction was precluded.

INFECTION OF MONKEYS BY THE INTRADERMAL INOCULATION OF THE VIRUS OF THE TSUTSUGAMUSHI DISEASE.

The inoculum was as described in the preceding paragraph. Two monkeys were used, one a gibbon and one a macacus; both reacted, and proved to be much more susceptible to the virus than were the rabbits.

In the gibbon, inoculation of infected aqueous humour at three sites on the abdominal wall resulted in the development of a macule on the 4th day at two sites, and a papule at the third site on the 11th day; the macules persisted for 2 days only and the papule for 4 days. At two other sites teased infected endothelium from Descemet's membrane was inoculated; and at both, papules developed on the 7th and 11th days that persisted for 9 and 4 days respectively; no necrosis ensued.

In the macacus monkey 6 inoculations were made, 2 with an inoculum of infected aqueous humour, 4 with an inoculum of infected endothelium; macule formation occurred at three sites, papule formation at all 6 sites on the 2nd and 3rd days; and, in 5 of these 6, necrosis ensued on the 5th to 7th days after inoculation (Fig. 14). The central necrotic scabs persisted for many days before being shed; 18 days after their first appearance, punched-out depressions marking their sites were clearly visible. The necrotic ulcers appeared identical with those described above in experimental rural typhus, and with the initial lesion of the tsutsugamushi disease in man. Control inoculations with an inoculum of aqueous humour and teased Descemet's membrane of normal rabbits were made at the same time, and yielded no trace of dermal reaction.

In both animals a febrile reaction occurred; in the gibbon it was of 6 days' duration, following an incubation period of 18 days; in the macacus monkey it was of 2 days' duration only, following an incubation period of 10 days (*vide* Charts V and VI). Swelling of the inguinal glands coincided with the development of necrosis in the case of the macacus, but was absent in the gibbon. Leucopenia was a feature of both infections, being especially marked in the gibbon, as the following figures show:

	(0).	(10).	(14).	(23).
Gibbon . .	14,500 .	12,500 .	6500 .	12,500
	(0).	(5).	(11).	(15).
Macacus . .	18,750 .	12,500 .	13,500 .	17,000

(Figures in brackets = number of days after inoculation.)

Neither animal appeared seriously ill during the infection.

In both the Weil-Felix reaction was positive, the " OXK " strain of *Proteus X.19* alone being agglutinated. The titres showed the waxing and waning features characteristic of active infection; in the gibbon the pre-inoculation titre of 1/17 rose to 1/3850 on the 28th day after inoculation; in the macacus monkey the initial titre of 1/50 rose to 1/385 on the 20th day.

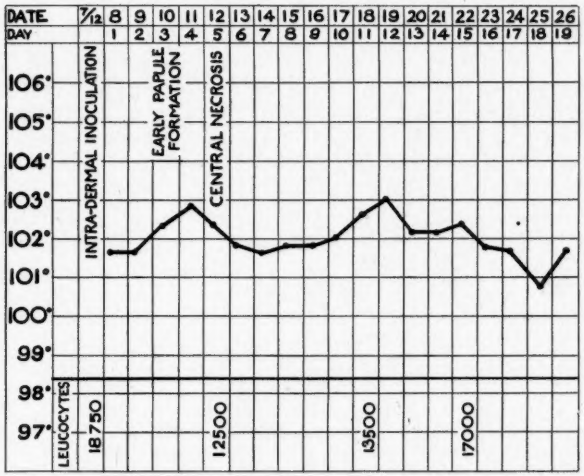

CHART V: TSUTSUGAMUSHI.—Monkey No. 3 (a macacus). Inoculated intradermally on December 7th, 1934, with infected material from the eye of a passage rabbit of a strain of the tsutsugamushi disease. For dermal lesions see Fig. 14.

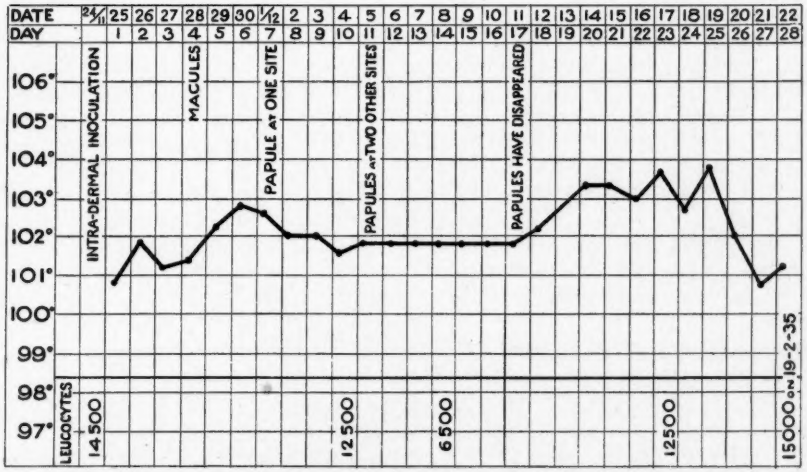

CHART VI: TSUTSUGAMUSHI.—Gibbon No. 1. Inoculated intradermally on November 24th, 1934. Source of inoculum, and strain, as for Monkey No. 3.

SUMMARY.

1. A strain of the rural form of tropical typhus has been established and maintained in guinea-pigs, and is now in its 97th generation. The history and characteristics of this strain are given. The clinical criterion of infection is a well-marked febrile reaction. Scrotal swelling does not occur. Ascites invariably follows intra-peritoneal inoculation of passage virus.

2. In more than one hundred other such attempts made in this laboratory it has proved impossible to maintain a strain beyond a few generations. Similarly all attempts to maintain the virus of the tsutsugamushi disease in guinea-pigs have failed. The guinea-pig must, therefore, be regarded as very insusceptible to the viruses of the rural typhus and tsutsugamushi group of fevers of Malaya.

3. Infection of rabbits by the intra-ocular inoculation of the virus of rural typhus and of the tsutsugamushi disease has been readily secured. The method, based on that of Nagayo *et al.*, and the criteria of infection, are described in detail.

4. The white rat is readily infected with the virus of rural typhus, the infection being of the " inapparente " form. That the virus could be maintained with unabated virulence for 21 generations indicates, by the criterion of Nicolle, that rural typhus is a murine strain.

5. Monkeys have been successfully infected by the intradermal route with the virus of rural typhus and of the tsutsugamushi disease. At the sites of inoculation, in the case of both viruses, necrotic ulcers have developed that appear to be identical with one another, and with the initial lesion of the tsutsugamushi disease in man ; in all other features the experimental infections in the monkey appear to be identical. Rabbits have been similarly infected.

6. The results of the Weil-Felix reactions of sera from rabbits and monkeys convalescent from experimental infection with rural typhus and the tsutsugamushi disease are summarized.

7. Methods of demonstration of *Rickettsia* from infected guinea-pigs and rabbits are described. In morphology, distribution and staining characteristics the *Rickettsia* demonstrable in material from animals infected with rural typhus and with the tsutsugamushi disease are identical, and do not appear to differ from the *Rickettsia orientalis* of Nagayo.

8. The experimental data secured indicate that, provided that intra-ocular inoculation is practised, the rabbit is the laboratory animal of choice in the case of the rural typhus and tsutsugamushi group of fevers (it being assumed that expense and scarcity make extensive use of monkeys impracticable). Further, these data stress the remarkable similarity of the behaviour of these two viruses in experimental laboratory animals—a similarity that, as will be set forth in a later paper, is fully supported by cross-immunity experiments.

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COLOURED PLATES.

(All preparations were stained by Giemsa's method.)

FIG. 1.—Guinea-pig 1635—a passage animal of the "Seerangayee" strain of rural typhus, maintained in guinea-pigs by the intra-peritoneal inoculation of virus. Shows smear of the fibrin covering the spleen. A group of mononuclear cells is seen; the cytoplasm of the cells contains numerous *Rickettsia*. Two features characteristic of the *Rickettsia* of rural typhus are readily seen, viz. the presence of two well-defined polar coccoid elements, in some instances slightly elongated, and the tendency of the organisms to occur in clusters close to the nucleus. × 1700.

FIG. 2.—Rabbit 600—a passage animal of the "Munian" strain of rural typhus, maintained in rabbits by intra-ocular inoculation of virus. Shows smear of scrapings from the endothelium covering Descemet's membrane in the reacting eye. *Rickettsia* are plentiful; their characteristics do not differ from those of the *Rickettsia* demonstrated in material from guinea-pigs infected with the virus of rural typhus. × 1800.

FIG. 3.—Rabbit 485—a passage animal of a series of rabbits infected with the virus of the "Seerangayee" strain of rural typhus. Maintained in guinea-pigs, this strain may be established in rabbits with ease by intra-ocular inoculation of heart-blood from an infected guinea-pig. Source of smear—as for Fig. 2. The typical clumping of *Rickettsia* close to the nucleus is well marked. × 2600.

FIG. 4.—Rabbit 1057—a passage animal of the "Kepong" strain of the tsutsugamushi disease, maintained in rabbits by the intra-ocular inoculation of virus. Source of smear as for Fig. 2. *Rickettsia* are plentiful; in morphology, distribution and staining characteristics they do not differ from those *Rickettsia* demonstrable in infected material from guinea-pigs and rabbits infected with the virus of rural typhus. × 1300.

PHOTOMICROGRAPHS.

FIG. 5.—Guinea-pig 2303—a passage animal of the "Seerangayee" strain of rural typhus. Shows smear of the fibrin covering the spleen. Three characteristics of the *Rickettsia* of rural typhus are well defined, viz. the presence of two polar elements, the more faintly staining inter-connecting rod, and the distribution within the cytoplasm as one or more clusters that lie close to the nucleus. × 1750.

FIG. 6.—Guinea-pig 2306—strain and source of material as for Fig. 5. Extra-cellular *Rickettsia* are abundant; other features noted above are well-marked. × 1600.

FIG. 7.—Rabbit 1144—a passage animal of a series of rabbits infected with the virus of the "Seerangayee" strain of rural typhus (*vide* notes on Fig. 3). Source of smear as for Fig. 2. Characteristic intra-cellular *Rickettsia* are seen. × 1600.

FIG. 8.—Rabbit 1055—a passage animal of the "Kepong" strain of the tsutsugamushi disease. Source of smear as for Fig. 2. Nucleus is somewhat degenerate. Intra-cellular *Rickettsia* are plentiful, and their morphological identity with the *Rickettsia* of rural typhus is evident. × 2000.

FIG. 9.—Rabbit 1163—a passage animal of the "Raub" strain of the tsutsugamushi disease, maintained in rabbits by the intra-ocular inoculation of virus. Source of smear as for Fig. 2. The typical clumping of *Rickettsia* at a point in the cytoplasm close to the nucleus is here well seen. × 1500.

FIG. 10.—Pneumococci in the blood of a mouse. × 1700. (This figure is given to afford comparison of the dimensions of pneumococci with those of the *Rickettsia* of rural typhus and the tsutsugamushi disease.)

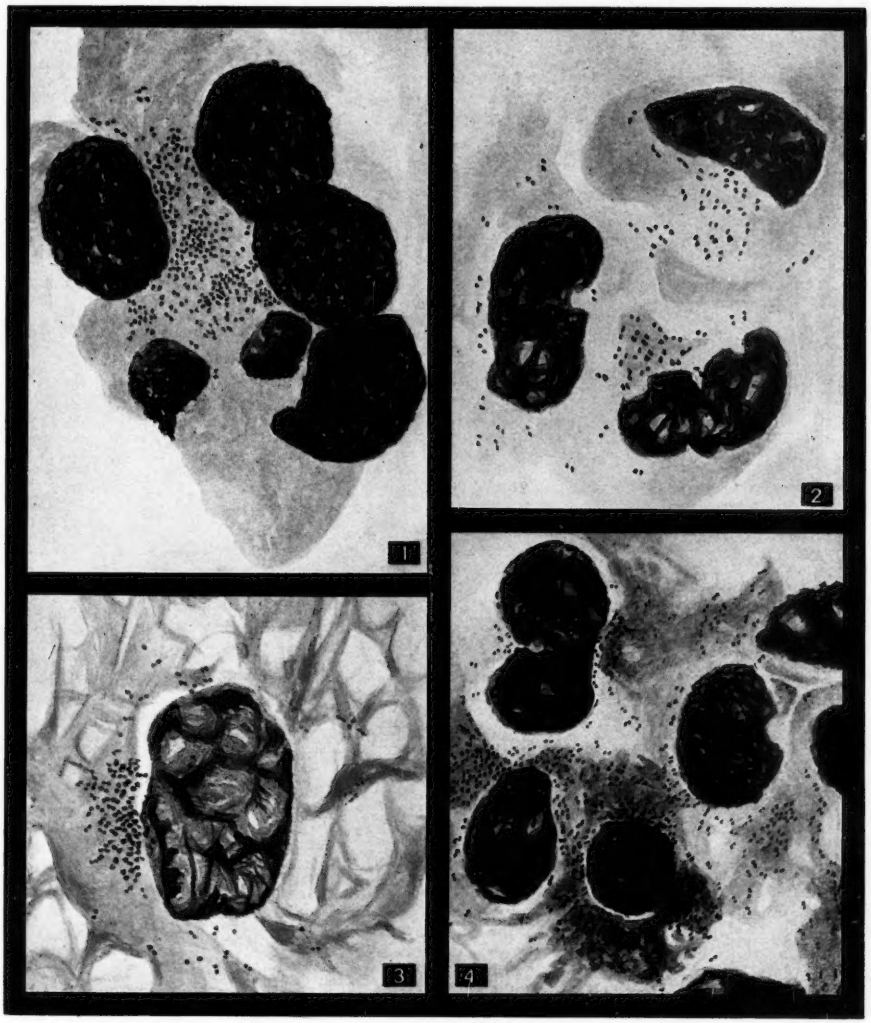
PHOTOGRAPHS.

FIG. 11.—Rabbit 1125—inoculated intradermally with material from the reacting eye of an infected rabbit of the "Seerangayee" strain of rural typhus. Shows the ulceration, with early necrosis, that followed inoculation, at two sites on each flank, of scrapings of the endothelium covering Descemet's membrane. Tenth day after inoculation. The less virulent aqueous humour from the same eye was inoculated intradermally at sites posterior to the above; and, at three of these sites, a feeble reaction is just perceptible—at the lower margin of the photograph.

FIG. 12.—Gibbon No. 2—inoculated intradermally with material from the reacting eye of an infected rabbit of the "Seerangayee" strain of rural typhus. Inoculations of both aqueous humour and teased endothelium from Descemet's membrane were made severally into the skin of both sides of the abdomen. This photograph, taken on the 12th day after inoculation, shows two small areas of necrosis at sites respectively above and below the umbilicus, to the right of the mid-line; the lower is the more distinct. On the left side there was development of papules that did not progress to the stage of necrosis. A large bubo in the left groin is prominent; the skin over it, tightly stretched and œdematous, has torn.

FIG. 13.—Monkey No. 4 (a macacus)—inoculated intradermally with material from the reacting eye of an infected rabbit of the "Seerangayee" strain of rural typhus. Photograph taken on the 12th day after inoculation. Shows five ulcers, each with well-marked central necrosis, in the skin of the abdomen at five sites of inoculation of teased endothelium from Descemet's membrane. Note the identity of these dermal lesions, caused by the virus of rural typhus, with those of Fig. 14, caused by the virus of the tsutsugamushi disease.

FIG. 14.—Monkey No. 3 (a macacus)—inoculated intradermally with material from the reacting eye of an infected rabbit of the "Raub" strain of the tsutsugamushi disease. Photograph taken on the 8th day after inoculation. Shows four ulcers, with well-marked central necrosis, in the skin of the abdomen at four sites of inoculation of teased endothelium from Descemet's membrane. (*Cf.* Fig. 13.)



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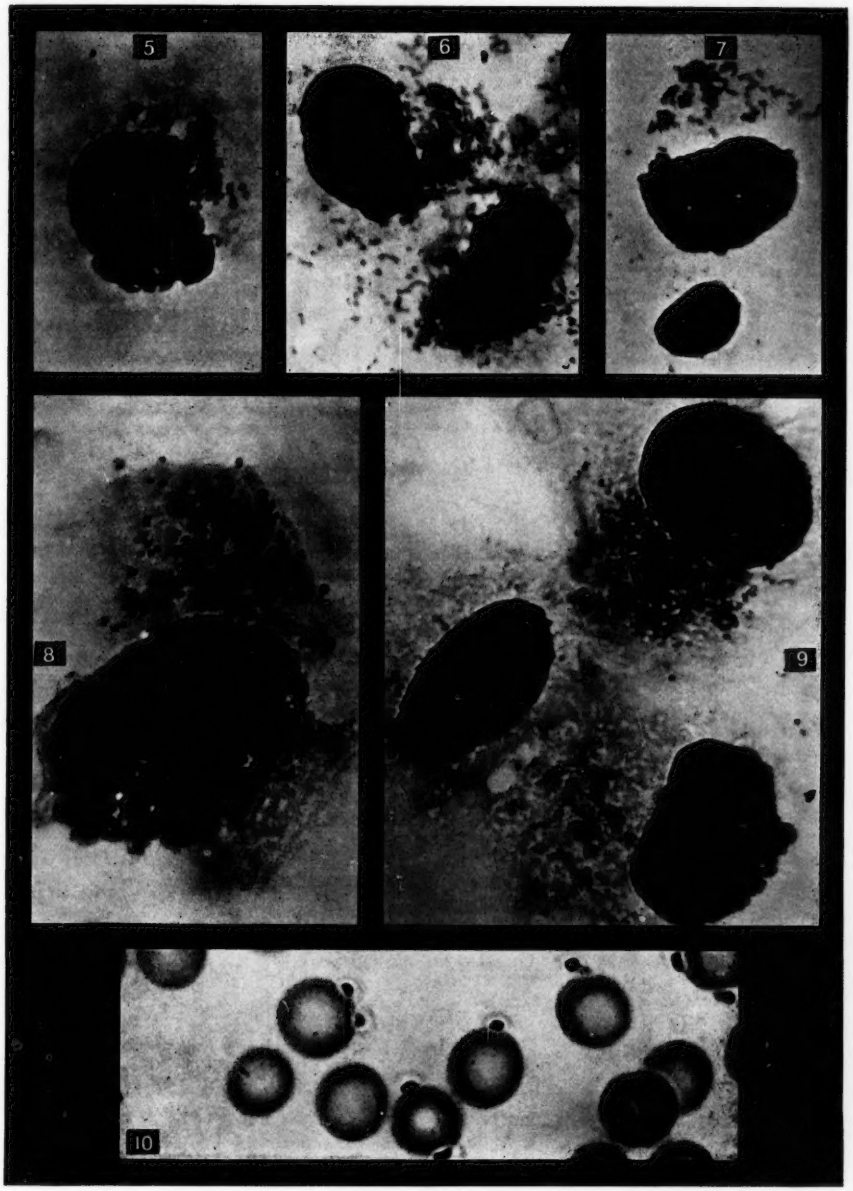

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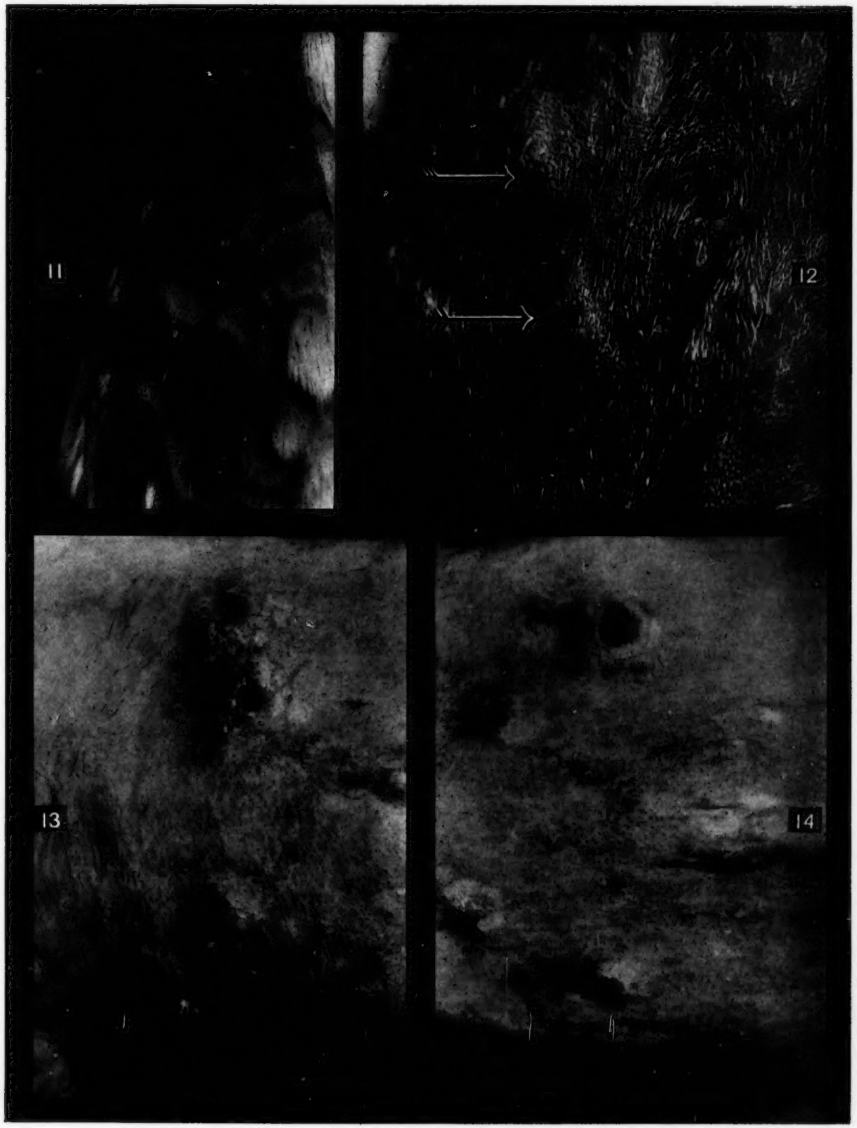

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THE TYPHUS GROUP OF DISEASES IN MALAYA. PART III: THE STUDY OF THE VIRUS OF THE URBAN TYPHUS IN LABORATORY ANIMALS.*

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LABORATORY tests correlated with statistics of domicile and occupation indicated to Fletcher and Lesslar (1926) at an early stage of their investigations that tropical typhus existed in Malaya in two forms, the rural form and the urban form. The latter is of rarer occurrence; in the five-year period 1929–1933 the rural cases numbered 768, the urban cases 204. In clinical features the two forms appear to be identical; in serological features they differ. Convalescent serum from cases of urban typhus agglutinates with characteristic waxing and waning titres the OX19 type of *Proteus X* strains, such as the "Warsaw" strain in use in this laboratory, but does not agglutinate the OXK type of *Proteus X* strains; in rural typhus the converse holds, convalescent serum agglutinating the OXK type but not the OX19 type.

A review of the occupations and domicile of those who have contracted tropical typhus affords another distinguishing feature between the two forms of the disease. Amongst the urban cases indoor workers largely preponderate; estate labourers and others who live and work in the country-side are few. Amongst the rural cases there are no indoor workers; all are connected with the country-side by domicile, work or recreation.

The suggestion of Fletcher and his collaborators (1929) that the virus of rural typhus is carried by a larval trombidiid has been strengthened by the more recent work of Gater (1930). In the case of urban typhus, on the other hand, the authors have recently demonstrated on three successive occasions the transmission of the virus by rat fleas (*X. cheopis*) under experimental conditions that approximated very closely to natural conditions; collateral experiments with the virus of rural typhus resulted in failure to effect transmission thus.

The major differential criteria enumerated above, and other minor ones, have been described in detail in sundry publications from this Institute (see references).

The scope of the present paper is restricted to a study of the virus of urban typhus, under experimental conditions, in the guinea-pig, white rat, rabbit and monkey.

Experiments demonstrating in the guinea-pig, rabbit, and monkey, the entire absence of cross-immunity between the virus of urban typhus and the virus of the group of fevers embracing rural typhus and tsutsugamushi disease will be described in a later paper in this series. Brief references to

* The "urban" form of tropical typhus is frequently referred to as "shop-typhus" and as "W-typhus". The first of these three designations will be adhered to in this series of papers, and for brevity will be written as "urban typhus".

the earlier of the findings recorded below were made in the Annual Reports of this Institute for the years 1932–3–4.

Before the present investigation, no study of the virus of urban typhus in laboratory animals had been undertaken. Since urban typhus has no focal distribution, and is of rarer incidence than is rural typhus, it occupies in clinical differential diagnosis a less prominent place than does rural typhus; diagnosis by the Weil-Felix reaction during convalescence is the rule, and hence an opportunity for obtaining infective blood from a patient is rare.

EXPERIMENTAL URBAN TYPHUS IN GUINEA-PIGS.

In August, 1932, we were enabled by the courtesy of Dr. H. P. Hodge, then in charge of the Tanglin Hospital, Kuala Lumpur, to have access to a patient in his care who was suffering from urban typhus. The patient, Manickam, was an indoor worker. He showed the typical clinical syndrome of tropical typhus; and his blood-serum subsequently gave high titres of agglutination with the OX19 type of *Proteus X* strains (1/3850 on the 18th day after onset of illness), but gave no agglutination of the OXK type.

Blood was drawn on the 6th day of fever, and inoculated by the intra-peritoneal route into two male guinea-pigs that had been fed on an ordinary diet. The animals reacted with fever on the 7th and 8th days after inoculation respectively, and with redness and swelling of the scrotum on the 8th and 9th days respectively. By inoculation into more guinea-pigs of exudate from the tunica vaginalis and of brain emulsion, taken from the two infected guinea-pigs at the height of febrile and scrotal reactions, infection was again secured. By similar inoculations the strain has been maintained by continuous passage in guinea-pigs, fed on an ordinary diet, without difficulty; and is now in its 109th generation. When the intra-peritoneal route of inoculation is used, less than 5 p.c. of guinea-pigs fail to react.

The length of the incubation period varies with the nature of the inoculum and the route of inoculation. By the intra-peritoneal route, inoculation of tunica exudate is followed by an incubation period of 5 to 7 days; inoculation of infected brain, spleen or heart blood by an incubation period of 6 to 9 days, prolonged occasionally to 12 days. Corresponding inoculations by the subcutaneous route result in a lengthening of the above periods by 2 to 4 days.

At the onset of fever, following intra-peritoneal inoculation, the temperature rises sharply during 2 days from the normal morning reading of 102° F., to reach a maximum morning level of 104·5° to 105·5° F.; fever continues without remission for 6 to 7 days, seldom having wider limits; defervescence is usually by quick lysis (Chart I). Occasionally the fever may range between 103° and 104° F. only, or may show a secondary rise; otherwise, little variation occurs. The febrile reaction is more constant in duration and degree than that of experimental rural typhus.

A scrotal reaction occurs in fully 95 p.c. of male guinea-pigs inoculated by the intra-peritoneal route with testicular exudate, and in about 70 p.c. of those thus inoculated with heart blood, or emulsions of brain or spleen. The reaction consists of a marked redness, tenderness and swelling of the

scrotum (Fig. 7). No necrosis of the skin has yet been observed. At the height of the reaction it is not possible to reduce the testes into the abdominal cavity; they reduce, however, under anæsthesia, unlike the testes in the scrotal reactions due to infections with *Spirillum minus* and *B. whitmori*. The reaction in the large majority of cases appears on the 1st and 2nd day of fever, less often on the 3rd day or the day prior to onset of fever. The duration of the scrotal signs varies; usually the fastigium is reached on the second day, and retrogression is prolonged until the 4th day; occasionally the evolution of the scrotal signs may occupy no more than one day. The reaction is bilateral in all but a few cases; where unilateral, post-mortem examination will usually reveal on the apparently unaffected side a degree of reaction in the tunica vaginalis, readily seen, though insufficient to have caused visible external changes in the scrotum.

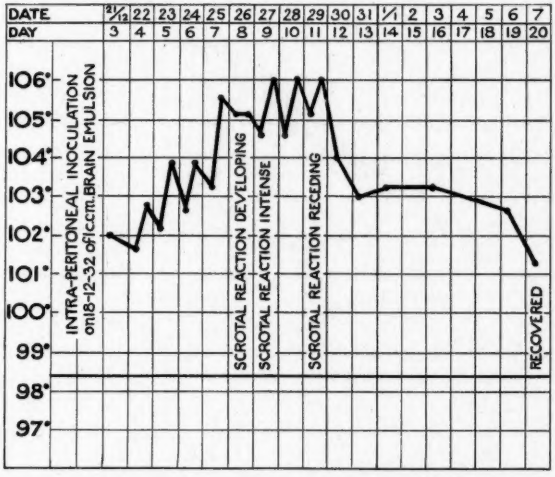

CHART I.—Guinea-pig 1230. A passage animal of the "Manickam" strain of urban typhus. Inoculated by the intra-peritoneal route.

During maintenance of the strain for the past three years by intra-peritoneal inoculation of brain emulsion, it has been noted that occasionally there are brief periods of non-occurrence of the scrotal reaction, following which the reaction recurs with its former constancy.

The mortality of guinea-pigs experimentally infected with the virus of urban typhus is less than 1 p.c.; it is doubtful if death ever occurs in the absence of intercurrent infection. After convalescence, guinea-pigs are immune to re-infection for a period of at least 6 months.

If the subcutaneous route of inoculation be used the febrile reaction is less intense (Chart II), and the proportion of guinea-pigs that fail to react is higher. Moreover, no scrotal reaction has yet been observed in any guinea-pig inoculated subcutaneously with the virus of urban typhus, indicating that this reaction is a local one between serous membrane and virus near the portal of entry.

Infected material from the tunica vaginalis forms the most potent inoculum, and may consist of physiological saline in which an excised testis has been shaken, or may be secured in greater potency by excising the testis complete with parietal tunica vaginalis, evaginating the testis, scraping both layers of the tunica vaginalis with the blade of a scalpel, and adding the scrapings to saline. The extreme virulence of the tunica exudate is attested by the fact that if one platinum-loopful of it is added to 5 c.c. of saline, and 2 c.c. of this extremely dilute emulsion is injected intra-peritoneally into a male guinea-pig, characteristic febrile and scrotal reactions ensue. The exudate is sterile on ordinary laboratory media.

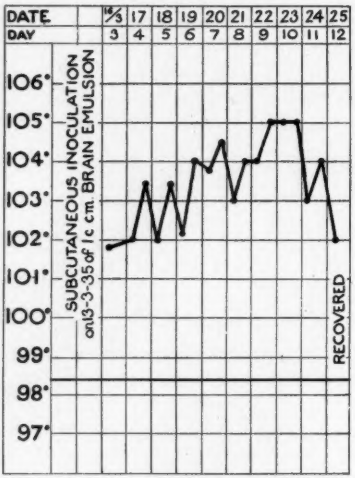

CHART II.—Guinea-pig 1240. A passage animal of the " Manickam " strain of urban typhus. Inoculated by the subcutaneous route.

The brain retains its virulence for at least 1 week after defervescence; no investigation to determine the limit of survival of virulence has been made. A number of infected brains, some in various strengths of glycerine, others dry, were frozen in ice, or kept at a temperature of 0°–5° C. for periods of 2 to 30 days, in order to determine the period of survival of virulence at these temperatures. Whereas control inoculations of the fresh brains were all positive, inoculation of the preserved brains showed non-survival of virulence for guinea-pigs. The method of preservation recommended by Laigret and Durand (1933), viz. to freeze material to a temperature of −10° to −12° C., has not been tried.

The two cardinal post-mortem signs are enlargement of the spleen, and a reaction that originates in the tunica vaginalis and extends to the adjacent tissues. Other less prominent and less frequent findings are congestion of the suprarenal glands, swelling of the lymphatic glands and hæmorrhages into or around them, and subpleural hæmorrhages. Ascites is never seen—a finding in sharp contrast to the post-mortem picture in experimental rural

typhus in guinea-pigs. The splenic enlargement is prominent from the 2nd day of fever onwards; the spleen is increased in size three or four times, and is deeply congested; its surface is smooth, contrasting with the coarseness of the splenic surface that is the usual finding in experimental rural typhus; a thin layer of fibrin occasionally covers the surface, but is less conspicuous and less frequently present than is the corresponding fibrinous deposit in experimental rural typhus. The macroscopic and microscopic changes involved in the characteristic scrotal reaction vary with the stage of the reaction. Post-mortem examination during the first day of the reaction shows swelling of the testis, injection of the visceral and parietal layers of the tunica vaginalis, and, covering the two layers, a viscid and slightly turbid exudate, in which here and there lie a few opaque flakes of fibrin. The polar fat is usually gelatinous in consistence, and often hæmorrhagic. External to the tunica vaginalis the scrotal tissues show a marked œdema. From the second day of the reaction onwards the exudate becomes more fibrinous and gluey, and may be stripped off in sheets. Permanent adhesions, such as commonly prevent reduction of the testis of guinea-pigs infected with *Spirillum minus* or *B. whitmori* convalescent from infection with the virus of Rocky Mountain spotted fever, are very rare in experimental infections caused by the inoculation of guinea-pig tissue virus. Usually, on the completion of convalescence, the scrotum and testis show no sign of previous affection. If, however, the inoculum used be an emulsion of infected rat fleas (*X. cheopis*), or a brain emulsion from an infected white rat, the resulting scrotal reaction will be much more intense, and permanent adhesions between the two layers of the tunica may develop, together with some degree of atrophy of the testis.

The microscopic findings likewise vary with the stage of the reaction. Smears of the very cellular exudate from the tunica vaginalis show that, as the macroscopic signs progress towards the fastigium, the polymorphonuclear cells predominate; they decrease in number as the signs retrogress, so that mononuclear cells, both macrophages and cells shed from the serous endothelium, then preponderate. Lymphocytes, eosinophiles and erythrocytes are present in small numbers. In smears made from tunica exudate during the scrotal reaction, and stained by Giemsa's method, the presence of *Rickettsia* may be demonstrated.

The histological findings in the brains of infected guinea-pigs are meagre. Hæmorrhagic extravasations from the smaller blood-vessels are occasionally seen. Less common are small localized areas of degeneration, free from nuclei, and staining coarsely. Typhus "nodes" are rare. As in the case of rural typhus, peri-vascular collections of mononuclear cells are readily demonstrable in preparations of fragments of mesentery spread onto slides, and stained by Giemsa's method.

The Demonstration of Rickettsia.

The *Rickettsia* of experimental urban typhus differ from those of experimental rural typhus in certain characteristics. The organisms are found within the cytoplasm of the endothelial cells that lie in the exudate on the tunica vaginalis and (occasionally) on the spleen of the reacting guinea-pig (*vide*

Figs. 1 to 5). Most of those in an infected cell are massed in one or more circumscribed areas, beyond which a few lie scattered irregularly. Occasionally extra-cellular forms are seen, that lie in close proximity to a heavily-laden cell, suggesting extrusion from a cell damaged by the excessive multiplication of *Rickettsia* within it (Fig. 3).

The organisms usually have the form of fine delicate bacilli. When stained by Giemsa's method the two ends are deep blue or purple, and the intermediate portion a paler blue. The more deeply stained ends may vary in length and width within fairly narrow limits; usually they appear as fine rods, tapering slightly at their proximal ends; occasionally they are set at an obtuse angle. The distance between the proximal ends may vary; if they are set wide apart, the intermediate faintly blue rod is distinctly seen; if they impinge on one another this rod may be barely visible or even invisible. Occasionally chain forms have been noted. Very rarely a single form is seen, appearing as one of the deeply stained polar components.

To facilitate appreciation of the dimensions of the *Rickettsia*, a photo-micrograph of a smear of pneumococci in the blood of a mouse, taken at a magnification of 1700, is included (Fig. 6). Considerable variation in length and width may occur, so that actual figures are only approximate. It may be stated that in average length the organisms vary between 1·0 to 2·5μ, and in width between 0·2 and 0·3μ. Variation in dimensions is in some instances more apparent than real; it may be due to some forms taking the stain more deeply than others, or to the two deeply staining proximal ends being set at an obtuse angle to one another, and so appearing at different depths of focus. To this variation in dimensions and the above suggested causes may, in part at least, be attributed the observation, frequently made, that in smears of the *Rickettsia* of rural typhus there will occasionally be found a few forms that resemble in morphology the *Rickettsia* of urban typhus; and conversely. Yet to the practised eye no difficulty presents itself on examination of smeared material in differentiating between the *Rickettsia* of the two forms of tropical typhus.

Much patience is required in the examination of a smear of infected material in order to find them, especially of smears from guinea-pigs infected with guinea-pig passage virus. Half-an-hour's search may prove fruitless. *Rickettsia* are more plentiful in material from guinea-pigs infected by the inoculation of an emulsion of infected fleas. The exudate and scrapings of the tunica vaginalis offer more hope of successful search than does the exudate that is occasionally present on the surface of the spleen.

The *Rickettsia* of rural typhus stain more readily and more deeply than the *Rickettsia* of urban typhus. The latter requre prolonged staining with Giemsa in buffered solution. The former can be stained in a few minutes by the Leishman stain, and buffering does not appear to be essential; tap water or distilled water give equally good results. The Castaneda stain has not proved satisfactory for either of the two *Rickettsia*.

To sum up, it may be said that the respective *Rickettsia* of rural and urban typhus are alike in the type of cell they infect, their intra-cellular distribution, and the differential staining given by Giemsa's method. They differ in length, width and numbers. In the *Rickettsia* of rural typhus the polar

components are unmistakably coccoid in shape; they are unmistakably linear in the *Rickettsia* of urban typhus.

We have compared the morphology, distribution and staining characteristics of the *Rickettsia* of urban typhus with those of *R. prowazeki*, present in smears from the tunica vaginalis of a guinea-pig infected with the virus of typhus exanthematicus; which smears, together with smears of *R. orientalis*, were very kindly furnished to us for comparative study by Prof. Nagayo. No difference in characteristics could be detected.

EXPERIMENTAL INFECTION OF WHITE RATS.

Owing to the great susceptibility of the guinea-pig to the virus of urban typhus, white rats have been little used, except in experiments relating to transmission. The brief account given here is based on observations of some twenty such infections. Following the intra-peritoneal inoculation of guinea-pig passage virus, an infection occurs that is of the " inapparente " form. Fever is either fitful or absent altogether; no scrotal swelling has been noted apart from one dubious reaction; the rats survive. Yet, if a rat is killed on the day coinciding with the fastigium of the infection of a control guinea-pig, inoculation of an emulsion of the brain or spleen into a guinea-pig will produce an intense infection. At post-mortem examination the only notable signs of infection are an enlargement and congestion of the spleen, the surface of which is smooth in contrast to the coarseness of the splenic surface in experimental rural typhus.

EXPERIMENTAL INFECTION OF RABBITS.

Experimental infection of rabbits was attempted by inoculation of infected material by three routes—intraperitoneal, intra-ocular and intradermal.

Eight rabbits were inoculated by the intraperitoneal route with infected material that consisted in 1 case of the blood of a patient, in 2 cases of an emulsion of brain, spleen and tunica exudate of a passage guinea-pig, and in 5 cases of tunica exudate only. No febrile or scrotal reaction developed. In 4 rabbits the Weil-Felix reaction was negative; in 4 it was positive, agglutination being only of the OX19 type of the *Proteus X* strains, and being of low titre.

The Attempted Isolation and Maintenance of Strains of Urban Typhus by the Intra-Ocular Inoculation of the Virus.

Using the technique described in Part I (p. 8), we have attempted to isolate and maintain the virus of urban typhus in rabbits. The results have differed greatly from those obtained with the virus of rural typhus.

On 26 occasions the virus of urban typhus was inoculated intra-ocularly into rabbits, the number used being 46. On 2 of these occasions the inoculum consisted of blood from the patient, Manickam; neither ocular reaction nor significant agglutination of *Proteus X* strains developed in the 4 rabbits that were inoculated, although the infectivity of the blood was shown by the intense febrile and scrotal reactions that developed in 2 guinea-pigs inoculated by the intraperitoneal route at the same time. On one occasion the inoculum consisted of an emulsion of fleas (*X. cheopis*) infected by feeding

on reacting guinea-pigs of the " Manickam " strain of urban typhus ; here, also, the 3 rabbits that were inoculated showed neither ocular nor serological reaction ; yet all 4 guinea-pigs inoculated intraperitoneally at the same time with the same emulsion yielded intense febrile and scrotal reactions. On 23 occasions the inoculum consisted of either heart blood or exudate from the tunica vaginalis, obtained from passage guinea-pigs of the " Manickam " strain at the height of reaction ; the number of rabbits inoculated in this first generation was 39 ; and of them 27 reacted with ocular signs (described below) sufficiently marked to justify attempts to maintain the virus further. From 23 of them aqueous humour was drawn at the height of reaction and passed into one or more rabbits, the total number thus inoculated, and constituting the second generation, being 28. Of these, only 9 gave ocular signs that justified further passage ; from all 9 aqueous humour was severally inoculated into 11 rabbits, that constituted the third generation. Of these 11, only 2 gave an ocular reaction ; and from them aqueous humour was inoculated into 3 more rabbits. All 3 of this fourth generation failed to give ocular signs. These findings are summarized in the following table :

Nature of inoculum.	Generation.	Number of rabbits inoculated.	Number of infections secured.	Percentage of infections secured.
Human blood	1st	4	0	(0)
Emulsion of fleas	1st	3	0	(0)
Heart blood or tunica exudate from guinea-pig	1st	39	27	(69)
				59
Aqueous humour	2nd	28	9	32
,, ,,	3rd	11	2	18
,, ,,	4th	3	0	0
Total		88	38	43

The above findings offer a marked contrast to the corresponding findings in the case of the viruses of rural typhus and the tsutsugamushi disease, both of which are viruses that, in a classification of the typhus-like fevers based on the antigenic type of virus, belong to the type " XK " (Felix and Heatly-Spencer, 1935). In Part I we have shown that from 12 patients with rural typhus seen early in the disease, it was possible to isolate 4 strains in rabbits by the intra-ocular inoculation of blood ; and that from time to time the " Seerangayee " strain in guinea-pigs has been transferred to rabbits with ease by the intra-ocular inoculation of infected heart blood. Similarly from 2 of 3 patients with tsutsugamushi disease, seen during the first week of fever, strains were thus isolated. Further, maintenance of the strains of both viruses by intra-ocular inoculation of infected aqueous humour afforded no difficulty.

No less striking than the above contrast is the similarity between the results relating to experimental urban typhus and corresponding results relating to the virus of typhus exanthematicus, recorded by Nagayo *et al.* (1930). Both are diseases, it may be noted, that, according to a classification based on the antigenic type of the virus, belong to the type X19. Reference to the table shows that, for experimental urban typhus, the percentage of

successful infections was 59 in the first generation, 32 in the second, 18 in the third and *nil* in the fourth. For typhus exanthematicus, Nagayo *et al.* give the corresponding percentages, in their series of 87 inoculated rabbits, as 88, 37, 31 and *nil*, the only difference in procedure being that they used " heart blood of guinea-pig or monkey ", where we have used heart blood and tunica exudate of guinea-pigs, human blood, and emulsions of fleas, in attempting to infect the first generation of rabbits. A degree of insusceptibility of the rabbit to infection, that increases with each succeeding passage and becomes absolute beyond the first few generations, is thus common to both experimental diseases.

In this connection it may be noted here, as will be reported more fully in a later paper, that very similar findings were obtained by the authors in attempts to maintain the virus of Rocky Mountain spotted fever in the rabbit by intra-ocular inoculation. A strain of this virus in ticks was received for study through the courtesy of Dr. R. R. Parker, Director of the Rocky Mountain Spotted Fever Laboratory, Montana, U.S.A. It was established in guinea-pigs, and maintained in them without difficulty ; but could not be maintained in rabbits beyond the second generation. Successive Weil-Felix reactions were carried out in 20 of the rabbits throughout convalvescence, Of them, 15 gave significant agglutination of the OX19 type of *Proteus X* strains, 5 gave negative results ; none gave any agglutination of the OXK type of *Proteus X* strains.

Tests were not carried out with the OX2 type of *Proteus X* strains, for which agglutinins are present in the sera of rabbits convalescent from Rocky Mountain spotted fever even more regularly than for the OX19 type (Davis, 1935) ; which finding serves to place this disease in a group distinct from urban typhus and typhus exanthematicus in a classification based on the antigenic type of the virus.

The Ocular Reaction.

The ocular reaction that results from inoculation of the virus of rural typhus and of the tsutsugamushi disease into the eye of a rabbit has been described in detail in the first paper of this series (p. 8). That caused by similar inoculation of the passage virus of the " Manickam " strain of urban typhus resembles this in evolution, a definite incubation period being followed successively by injection of the circumcorneal vessels, of the vessels of the iris, turbidity of the aqueous humour, and, lastly, marking the beginning of retrogression, the appearance of pannus. But each phase of the reaction is of shorter duration, and of less intensity, than in infections due to the viruses of strains of rural typhus and the tsutsugamushi disease. The evolution of the ocular reaction in successful infections of rabbits with the virus of Rocky Mountain spotted fever was closely similar to that occurring in experimental urban typhus.

The Demonstration of Rickettsia.

If, from a rabbit infected with the virus of urban typhus by the intra-ocular route, the reacting eye be excised when signs are maximal, and smears be made of scrapings from the endothelium lining Descemet's membrane,

and stained by Giemsa's method, *Rickettsia* may be demonstrated. The organisms are, however, much fewer in number than are those of *R. orientalis* in corresponding smears from rabbits infected experimentally with the viruses of rural typhus and the tsutsugamushi disease. In the experience of the authors they are even more difficult to find in the eye of a rabbit than in the tunica exudate of a reacting guinea-pig. When found, they do not differ from those demonstrable in material from infected guinea-pigs.

The Weil-Felix Reaction Following Intra-Ocular Infection.

The Weil-Felix reactions obtained in rabbits infected with the virus of urban typhus will be reported in detail in a later paper, and are given here in brief only. From 58 rabbits inoculated by the intra-ocular route, successive samples of sera, drawn throughout convalescence, were submitted for the Weil-Felix reaction. Twenty-eight sera proved to be negative, and 30 to be positive. Of these 30, maximum titres ranged between 1/40–1/200 in 19, 1/200–1/400 in 6, 1/400–1/800 in 3, while in 2 the titres were 1/1700 and 1/1925 respectively. Agglutination was in every case of the OX19 type of *Proteus X* strains; in no case did any agglutination of the OXK type occur.

There was no relation between the source of inoculum and the height of titre obtained. An inoculum of a flea emulsion, proved to be highly virulent for guinea-pigs, failed to stimulate any development of agglutinins in the sera of the 3 rabbits inoculated with it. Heart blood, tunica exudate and infected aqueous humour gave much the same proportion of successes and failures.

The concurrence of the two criteria of infection in a reacting rabbit, viz. ocular reaction and development of agglutinins, was usual. Thus, of the 30 rabbits the sera of which gave a positive Weil-Felix reaction, 26 also developed ocular signs; of the 28 rabbits the sera of which gave a negative Weil-Felix reaction, 22 failed to develop ocular signs.

Only four attempts to infect rabbits by the intradermal route were made; the results are reported below. There has been no mortality in rabbits due to infection with the virus of urban typhus by any of the above three routes of inoculation.

ATTEMPTS TO INFECT MONKEYS BY THE INTRADERMAL INOCULATION OF VIRUS.

In order to see if it were possible to produce a necrotic ulcer such as that developing in rural typhus and tsutsugamushi disease (pp. 12, 18) in experimental infections with urban typhus, two macacus monkeys were inoculated intra-dermally at multiple sites with tunica exudate from a reacting guinea-pig of the "Manickam" strain of urban typhus. In both cases, to prove the virulence of the inoculum, a guinea-pig was inoculated intra-peritoneally; both reacted with febrile and scrotal reactions. In the first monkey a mild infection resulted. Fever was present only on the 5th and 6th days after inoculation, and, as a low secondary rise, from the 11th to the 18th days; agglutinins for the OX19 type of *Proteus X* strains rose gradually from

zero to give a titre of 1/96 on the 15th and 19th days after inoculation respectively, and thereafter declined; there was no significant agglutination of the OXK type. No local lesion developed at the site of inoculation, and there was no leucopenia. In the second monkey a mild infection resulted also; febrile reaction occurred only on the 11th and 12th days after inoculation; agglutinins for the OX19 type of *Proteus X* strains rose by steps from zero to reach a titre of 1/110 on the 12th and 19th days respectively, and thereafter declined; no significant agglutination of the OXK type developed. No local lesion occurred at the site of inoculation. There was a moderate leucopenia, the number of leucocytes falling from an initial count of 17,000 to 11,000 on the 16th day, and rising to 18,750 on the 28th day.

In the gibbon, an infection resulted, but was mild only. Febrile reaction occurred on the 7th day, and lasted for four days. The Weil-Felix reaction was positive; agglutinins for the OX19 type of *Proteus X* strains rose from zero to give a maximum titre of 1/220 on the 20th day after inoculation; there was no significant agglutination of the OXK type. No local lesion developed at the site of inoculation. There was a marked leucopenia, the number of leucocytes falling from an initial count of 15,000 to 6,250 on the 8th day, and rising to 18,000 on the 20th day.

Owing to the scarcity of available monkeys, this series of experiments could not be extended. These three results, however, find corroboration in the results of inoculation, on two separate occasions, of infected material into four rabbits by the intradermal route. In none did any local lesion develop—a result that is in sharp contrast with the result of similar inoculations of the virus of rural typhus and the tsutsugamushi disease (*vide* Parts I and II, pp. 9, 17).

SUMMARY.

1. A strain of the urban form of tropical typhus has been established in guinea-pigs, and maintained in them for more than one hundred generations. The history and characteristics of the strain are given. The clinical criteria of infection are febrile and scrotal reactions.

2. Methods of demonstration of *Rickettsia* in material from infected guinea-pigs and rabbits are described. In morphology, distribution and staining characteristics these *Rickettsia* do not appear to differ from *R. prowazeki*.

3. The infection of rabbits by intra-ocular inoculation of virus has met with only partial success; the strains rapidly lose virulence, and do not survive beyond the third generation. The results are closely similar to those reported by Nagayo *et al.*, in corresponding infections of rabbits with the virus of typhus exanthematicus, and to those obtained by the authors in corresponding infections with a strain of Rocky Mountain spotted fever.

4. Infection of white rats has been readily secured, and has been of the "inapparente" form.

5. Two monkeys, inoculated intradermally with infected material, showed a mild general reaction only; no lesion developed at the site of inoculation.

6. The results of the Weil-Felix reactions of sera from rabbits and monkeys

convalescent from the infection are summarized. Agglutination is of the OX19 type of *Proteus X* strains, never of the OXK type.

7. The experimental data obtained indicate that the guinea-pig is the laboratory animal of choice for the study of the urban form of tropical typhus.

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COLOURED PLATES.

(All preparations were smears, and were stained by Giemsa's method.)

FIG. 1.—Guinea-pig 2016, a passage animal of the "Manickam" strain of urban typhus. Shows an endothelial cell in the exudate from the tunica vaginalis. Two features characteristic of *Rickettsia* are well marked, viz. bi-polar staining with Giemsa's method, and their distribution within the cytoplasm as clusters, beyond which lie a few individuals scattered diffusely. Compared with the *Rickettsia* of rural typhus and the tsutsugamushi disease (*vide* Parts I and II of this series), the *Rickettsia* of urban typhus are longer and narrower; the polar components in the former approximate to a coccoid form, in the latter to a linear form. × 1700.

FIG. 2.—Guinea-pig 2120, a passage animal of the 75th generation of the "Manickam" strain of urban typhus. Shows an endothelial cell in the exudate from the tunica vaginalis; within the cytoplasm lie *Rickettsia* massed as a corona. × 1800.

(For photomicrograph see Fig. 5.)

FIG. 3.—Guinea-pig 2187, infected by intra-peritoneal inoculation of an emulsion of rat fleas (*X. cheopis*); these had fed on white rats infected by inoculation of passage material from a reacting guinea-pig of the "Manickam" strain of urban typhus. Shows a bi-nuclear endothelial cell in the tunica exudate; the cytoplasm of the cell is laden with *Rickettsia*. Extracellular *Rickettsia* are also seen, some of them arranged in chain-formation. × 1800.

PHOTOMICROGRAPHS.

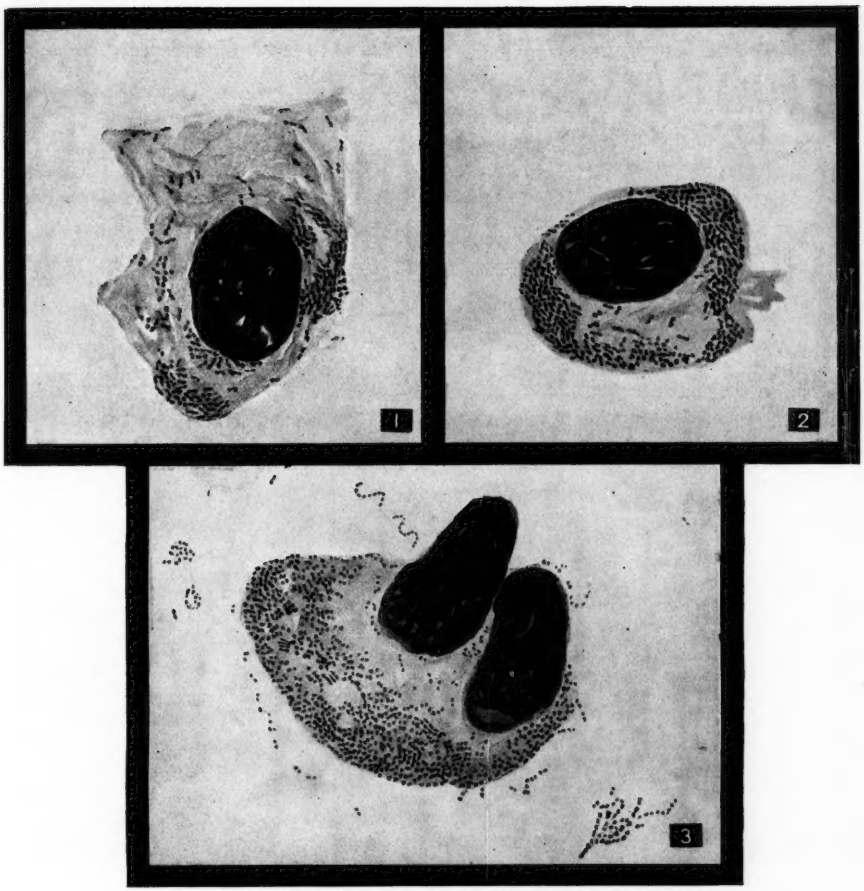
(All preparations were smears and were stained by Giemsa's method.)

FIG. 4.—Guinea-pig 2187. Photograph of an endothelial cell in another smear of the tunica exudate from this guinea-pig (*vide* Fig. 3). The cytoplasm contains an abundance of *Rickettsia*. The linear morphology of the *Rickettsia* of urban typhus is here well illustrated. × 1600.

FIG. 5.—Guinea-pig 2120. Photomicrograph of the smear preparation depicted in Fig. 2. × 1900.

FIG. 6.—Photomicrograph of pneumococci in the blood of a mouse. × 1700. (This figure is given to afford comparison of the dimensions of pneumococci with those of the *Rickettsia* of urban typhus.)

FIG. 7.—Experimental urban typhus. The scrotal reaction that follows the inoculation of a guinea-pig with infected material by the intra-peritoneal route.



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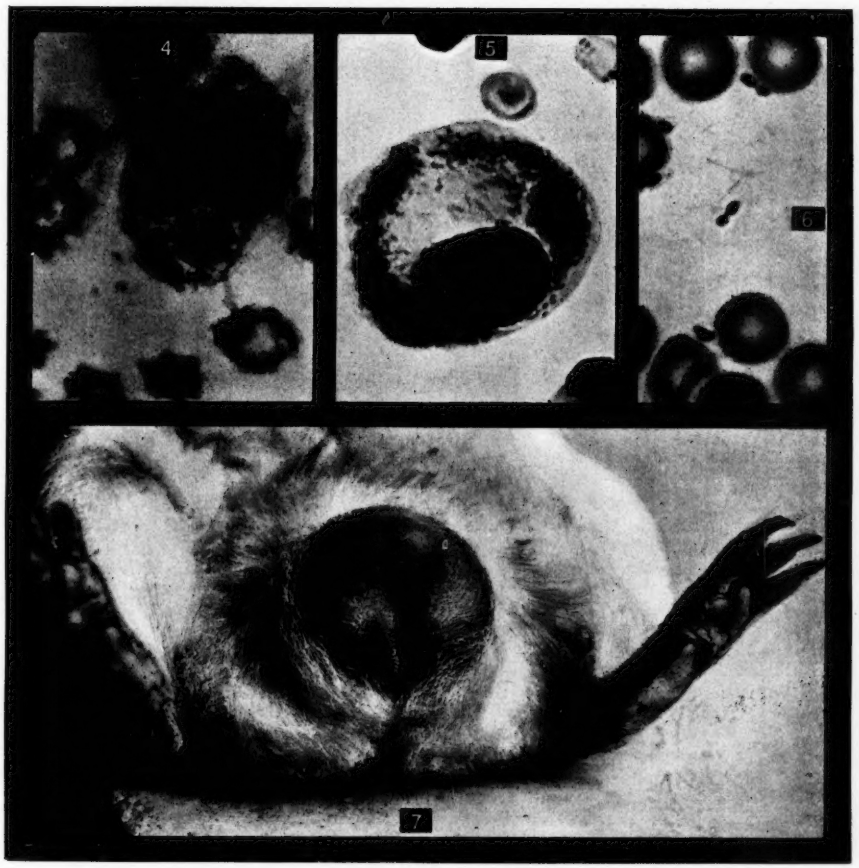

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DETERMINING THE GROUP OF HUMAN BLOOD-STAINS: NOTES ON AN ANOMALOUS GROUP O SERUM.

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THE observations reported in this communication were made during the course of an investigation of the grouping of dried blood-stains on cloth by the technique of Martley and Roche Lynch (1923, 1928). Briefly this method consists of two parts:

A. *Examination of the stain for agglutinins.*—The stain is extracted with saline and the extract is tested for the presence of agglutinins. Positive results, *i. e.* the demonstration of agglutinins, are reliable; negative results, which indicate absence of agglutinins, should not be relied on alone, but are of importance when confirmed by the agglutinin absorption test.

B. *Examination of the stain for agglutinogens.*—An O serum is absorbed with the stain, the α and β agglutinin titres being measured before and after such absorption. Absence or marked reduction of an agglutinin following absorption indicates the presence of the corresponding agglutinogen in the stain. The agglutinogens are relatively stable substances and can be demonstrated in stains that have been preserved for long periods.

Table I gives the details of a typical grouping experiment.

TABLE I.—*Examination of Blood-Stains on Cloth for Agglutinins and Agglutinogens.*

Technique: ½ sq. cm. of each stain and of unbloody cloth shredded fine with scissors and put into two sets of small tubes; 35 c.mm. normal saline or O serum added to each tube and soaked into the cloth fibres by prodding with a fine glass rod; tubes connected to water-pump and pressure reduced; pressure released and process repeated (to get rid of air-bubbles and bring fibres into intimate contact with the fluid); tubes stoppered and left at 5° C. overnight. Next day fluids withdrawn by capillary pipettes and centrifuged. Unit volumes (approx. 6 c.mm.) saline extracts mixed with unit volumes A and B corpuscles (2 p.c. suspension in saline) on glass microscopic slides. Unit volumes sera, diluted 1-2, 1-4 . . . 1-256, placed on slides, four dilutions per slide (separated by grease pencil marks) and unit volumes corpuscles added; mixed with platinum wire and placed in moist chambers (Petri dishes with moist blotting-paper in the bottom and wooden laths for slides to rest on) for 30 mins. Slides gently rocked and reactions examined microscopically with a 2/3 in. objective.

Saline extract of—	Corpuscles. A.	Corpuscles. B.
Stain 8	+	+
,, 9	—	+
,, 10	—	—
,, 11	+	—
Unbloody cloth	—	—

TABLE I—*continued.*

O serum absorbed with unbloody cloth.

Corpuscles.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.
A	+	+	+	+	+	+	±	−
B	+	+	+	+	±	−	−	−

O serum absorbed with Stain 8.

A	+	+	+	+	+	+	±	−
B	+	+	+	+	±	−	−	−

O serum absorbed with Stain 9.

A	±	−	−	−	−	−	−	−
B	+	+	+	+	−	−	−	−

O serum absorbed with Stain 10.

A	±	−	−	−	−	−	−	−
B	+	+	+	+	−	−	−	−

O serum absorbed with Stain 11.

A	+	+	+	+	+	+	±	−
B	±	−	−	−	−	−	−	−

+, ± and − signify complete or marked, slight, and absence of agglutination respectively.

	Agglutinin.	Agglutinogen.	Group is therefore—
Stain 8 contains	a β	—	O
,, 9 ,,	β	A	A
,, 10 ,,	—*	A	A
,, 11 ,,	a	B	B

* β agglutinin titre of serum 10 was low, hence failure to demonstrate agglutinin in the extract of the dried stain.

These results were confirmed by examining the fresh corpuscles and sera of individuals 8, 9, 10 and 11.

In the process of examining stains of known group with a variety of O sera, one serum (" H ") was found which gave results which differed from those given by a normal O serum. The use of this serum for absorption led to Group A stains being labelled AB, while B and O stains were classified correctly. Experimental details are shown in Table II. This suggested that agglutinogen A removed practically all the β agglutinin as well as the a agglutinin from O serum " H ", but that agglutinogen B removed agglutinin β only.

TABLE II.—*Absorption of O Sera " H " and " L " with Stains of A, B and O Bloods.*

	O SERUM " H ".								O SERUM " L ".							
Absorbed with unbloody cloth.																
Corpuscles.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.
A	+	+	+	+	+	+	±	−	+	+	+	+	+	+	+	−
B	+	+	+	+	−	−	−	−	+	+	+	+	±	−	−	−

TABLE II—*continued.*

Absorbed with O stain.

A	.	+	+	+	+	+	+	±	−	+	+	+	+	+	+	±	−
B	.	+	+	+	+	−	−	−	−	+	+	+	+	±	−	−	−

Absorbed with A stain.

A	.	±	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
B	.	+	−	−	−	−	−	−	−	+	+	+	+	±	−	−	−

Absorbed with B stain.

A	.	+	+	+	+	+	+	−	−	+	+	+	+	+	+	±	−
B	.	±	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−

On the basis of the above tests :

O stain is of group			O				O
A	,,	,,	,,	AB			A
B	,,	,,	,,	B			B

Experiments were then carried out on the absorption of serum " H " and other O sera with fresh corpuscles of groups A and B. The results are set forth in Table III. These confirmed the above findings that the absorption of serum " H " with B corpuscles was purely homologous, but that absorption with A corpuscles produced in every case a marked reduction of the heterologous agglutinin in addition to removing the homologous agglutinin.

TABLE III.—*Absorption of O Sera " L " and " H " with A and B Corpuscles.*

Technique : 1 unit volume packed washed corpuscles added to 4 unit volumes serum, mixed and left at room temperature for 3 hours ; centrifuged ; supernatants put up with 2 p.c. suspension corpuscles as before.

		O SERUM " L ".								O SERUM " H ".								
		1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	Tested with—
Unabsorbed		+	+	+	+	+	−	−	−	+	+	+	+	+	±	−	−	
Absorbed with A corpuscles	1	+	+	+	+	±	−	−	−	+	−	−	−	−	−	−	−	B corpuscles.
	2	+	+	+	+	±	−	−	−	±	−	−	−	−	−	−	−	
	3	+	+	+	+	+	−	−	−	+	−	−	−	−	−	−	−	
	4	+	+	+	+	±	−	−	−	+	−	−	−	−	−	−	−	
	5	+	+	+	+	+	−	−	−	±	−	−	−	−	−	−	−	
	6	+	+	+	+	+	−	−	−	±	−	−	−	−	−	−	−	
	7	+	+	+	+	+	−	−	−	+	−	−	−	−	−	−	−	
	8	+	+	+	+	±	−	−	−	+	−	−	−	−	−	−	−	
Unabsorbed		+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−	A corpuscles.
Absorbed with B corpuscles	1	+	+	+	+	+	+	±	−	+	+	+	+	+	+	+	−	
	2	+	+	+	+	+	+	+	−	+	+	+	+	+	+	±	−	

N.B.—Homologous agglutinins were completely removed in all the absorptions.

Discussion.—There are a few scattered references in the literature to absorption of O sera with A or B corpuscles leading to diminution of the heterologous agglutinin. Landsteiner and Witt (1926) state: " . . . after the absorption

of sera O with a blood A or B there results often, *but not regularly*, a considerable loss of activity for bloods B and A respectively ". It has been suggested that this phenomenon is due to a partial linkage of α and β agglutinins in certain O sera. The outstanding points of interest in the experiments with O serum " H " detailed above are the regularity with which β agglutinin is removed by absorption with A corpuscles, and the complete absence of any similar effect on the α agglutinin by absorption with B corpuscles. Presumably this must be due to some form of " linkage " between the α and β agglutinins in serum " H ", which functions in one direction only, *i. e.* from A→B, but not from B→A. The removal of β agglutinin by absorption with A corpuscles was in no case quite complete, so probably a small portion of the β agglutinin was free of any " attachment " to the α agglutinin.

The significance of the above phenomenon in relation to the medico-legal examination of blood-stains is obvious ; the O serum employed for absorption must not show any heterologous absorption with A or B corpuscles. Some authorities advise separate absorptions with α and β sera or the employment of mixed α and β sera instead of O serum, but the writer has found the latter entirely satisfactory provided the O serum shows only homologous absorption with known A and B corpuscles.

It is a pleasure to express my thanks to my colleague, Dr. F. C. Martley, for his valuable assistance and kindly interest in this research.

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IMMUNOLOGICAL STUDY OF TYPHOID POLYSACCHARIDE.

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FURTH and Landsteiner (1928, 1929) and White (1928, 1931) made it clear that at the surface of typhoid and paratyphoid bacilli there are present carbohydrate components, which determine the bacterial virulence and antigenic behaviour. The antigenic apparatus of the somatic part of typhoid bacilli, according to White (1931, 1932, 1933) consists of the following substances : A specific polysaccharide, situated on the surface of bacterial cells of S form ; a polysaccharide (R), situated at the surface of bacterial cells of R form ; more deeply located polysaccharides (ρ_1 and ρ_2) ; a protein (T), soluble in acid 75 p.c. alcohol ; and a protein (Q), soluble in acid 95 p.c. alcohol.

The purpose of the present work is to study the antibody corresponding to the typhoid polysaccharide of S form.

Avery and Goebel (1931) prepared a synthetic antigen by linking Type III pneumococcal polysaccharide to horse-serum globulin ; with this they immunized rabbits. The antisera thus obtained protected mice against pneumococcus Type III, but not against Types I and II. Zozaya (1932) and Zozaya and Clark (1933) immunized animals with the polysaccharides of pneumococci, adsorbed on collodion particles, charcoal, aluminium hydroxide or casein. Jacobs (1934) obtained an immunological response with the polysaccharide of *V. cholerœ*, adsorbed on particles of charcoal.

Preparation of Typhoid Polysaccharide.

The culture of the preparation of typhoid polysaccharide was strain 203, from the collection of the Kiev Bacteriological Institute. This strain was in its morphological, cultural, physico-chemical and immunological properties of typical S form. Agar cultures were washed off with distilled water, and the suspensions were boiled for one hour after the addition of 25 p.c. saturated NaOH. From the solution thus prepared the polysaccharide was precipitated by alcohol and purified as follows : The precipitate was dissolved in 1 p.c. acetic acid and re-precipitated by alcohol ; it was then dissolved in 1 p.c. NaOH and again precipitated by alcohol ; this procedure was repeated four to five times. Then the precipitate was dissolved in physiological saline solution and was again precipitated ; the final precipitate was dried. The polysaccharide gave none of the usual reactions for proteins. Injected into animals it did not stimulate the formation of antibodies, and it may be characterized as a hapten.

Immunization.

The precipitate obtained from the interaction of the typhoid polysaccharide with immune serum was used as a synthetic antigen. The polysaccharide was mixed in optimal dilution with immune rabbit- or horse-serum. The precipitate which formed was separated by centrifugation and, after being washed in physiological saline, was suspended either in that solution or in the original supernatant fluid. The suspensions were injected intravenously into rabbits. One group of rabbits on three successive days received 1 mg., 2 mg. and 2 mg. of the precipitated polysaccharide. Another group of rabbits received five injections of 2 mg. to 4 mg. every sixth day. From all rabbits, before the beginning of immunization blood was taken for precipitation tests with typhoid polysaccharide ; the tests were negative with 1 : 1000 dilution of polysaccharide.

During the period of immunization the rabbits did not show any decrease in weight or rise of temperature. On the tenth day after the last injection the animals were bled.

Rabbits 114, 117 and 119 received injections of polysaccharide, precipitated with rabbit immune serum ; all the others with horse-serum. Rabbits 118 and 119 were immunized with washed precipitates, suspended in physiological saline. All the rest were immunized with suspensions in the original supernatant.

Precipitation.

Sera were examined for precipitins against purified polysaccharide by the ring test. As shown in Table I, three injections of small quantities of the precipitated polysaccharide produced detectable antibodies. After more intensive immunization precipitins were formed in considerable quantities.

TABLE I.—*Precipitation.*

Rabbit No.	Number of injections.	Quantity of injected polysaccharide in mg.	Maximal dilution of polysaccharide.
114	3	5	2000
117	3	5	2000
118	3	6	..
119	3	5	..
128	3	5	..
201	5	12	10,000
202	5	15	..
203	5	12	10,000
204	5	12	8000
205	5	12	5000
206	5	15	10,000
207	5	15	10,000
208	5	20	5000
209	5	12	..

The lowest dilution of polysaccharide tested was 1 : 2000. The result of the precipitation reaction was noted after twenty minutes at the temperature 17°–18° C.

Agglutination.

For agglutination tests the following typhoid antigens were used :

1. Strain 203—live suspensions.
2. Strain H901* from the Lister Institute.
3. Alcohol suspension of strain 203 prepared by Bien's method for the estimation of somatic agglutinins.
4. Strain O901* from the Lister Institute.

The sera were taken from 10 rabbits subjected to the more intensive immunization. Table II shows that the immunization of rabbits with precipitated typhoid polysaccharide leads to the formation of typhoid agglutinins. The titre of agglutination with the culture 203 treated by Bien's method and with a non-motile variant O901 is much higher than with live cultures 203 and H901.

The agglutinins were absorbed from such of the sera which agglutinated Strain O901 with organisms of this strain. The absorbed sera were then tested with Strains 203 and H901 ; they all failed to show any agglutinations in dilution 1 : 50.

* Strains H901 and O901 were received from the Lister Institute owing to the kindness of Dr. A. Felix. We avail ourselves of the opportunity to express here once more our deep gratitude to Dr. Felix for always kindly attending to the interests of our Institute.

TABLE II.—*Agglutination Titres of Sera of Rabbits Immunized with Polysaccharide.*

Rabbit No.	Culture.			
	203 live.	H901.	203 treated by Bien.	O901.
201	800	..	3200	..
202	400	100	1600	200
203	800	..	800	50
204	800	..	3200	..
205	1600	..	800	200
206	200	..	3200	..
207	1600	200	800	800
208	1600	200	3200	1600
209	1600	400	3200	800
210	400	200	3200	400

DISCUSSION.

The results recorded prove that the polysaccharide of *Bact. typhosum* precipitated with immune sera stimulated in animals the formation of antibodies capable of precipitating the purified polysaccharide and agglutinating suspensions of *Bact. typhosum*. The much higher agglutination titres obtained with strain 203, treated by Bien's method and with the non-motile Strain O901, show that the agglutinins react with the somatic part of bacteria. Absorption tests also showed that the sera contained only O-agglutinins and had no H-agglutinins.

The method of preparing a synthetic antigen described here is much simpler than linking the polysaccharide chemically to some protein molecule, and the resultant antigen is more efficient in stimulating the production of antibodies than are polysaccharides adsorbed on colloids. It is therefore probable that the use of precipitated polysaccharides as antigens would throw light on the immunological significance of various bacterial carbohydrate substances.

SUMMARY.

1. The precipitate resulting from the interaction of *Bact. typhosum* polysaccharide and anti-typhoid serum produces, when injected into animals, anti-polysaccharide immune bodies.

2. The immune sera not only precipitate with purified polysaccharide, but also agglutinate suspensions of *Bact. typhosum*.

3. The agglutinins react with the somatic part of the bacteria.

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THE DETECTION OF ANTIGENIC DIFFERENCES IN MOUSE ERYTHROCYTES BY THE EMPLOYMENT OF IMMUNE SERA.

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In a previous paper evidence was brought forward to show that it was possible to differentiate at least two antigenic types of mouse corpuscles by means of normal human group A serum, and that the difference between the two types of reaction was determined by a single gene (Gorer, 1936). The present communication deals with differences detectable by means of sera from immunized rabbits.

MATERIALS AND METHODS.

The mice used in these experiments came from strains that had been brother-sister inbred for over 25 generations. In describing strains of mice it is very important to state the system of breeding employed. A strain that has been pen inbred for some years may frequently be shown to be heterozygous for easily recognizable genes, whilst after about 20 generations of brother-sister mating a close approximation to a genetically homogeneous stock is obtained. Since antigenic differences have not been shown to arise except as the result of genetic differences, at any rate in healthy tissues, it will be seen that there are good grounds for regarding a genetically homogeneous stock as antigenically homogeneous.

The three following strains were used in the experiments to be described: (1) *An albino strain.* The strain A of the Roscoe Jackson Memorial Laboratory which has been used extensively in work on the genetics of tumour transplantation. (2) *An agouti* or wild type strain. The CBA stock of the Roscoe Jackson Laboratory. Has a low incidence of spontaneous tumours. (3) *A strain of black non-agoutis* from Dr. Dunn's laboratory at Columbia University. These strains had all been brother-sister inbred for over 20 generations before being imported by Prof. J. B. S. Haldane in 1932, and have since been exclusively mated brother to sister.

Rabbits were drawn from stock, thirteen being used in all. Three were immunized with the blood of black mice, four with the blood of albinos, and six with the blood of agoutis. One rabbit died during the immunization, but sera were obtained from all the others.

Injections, etc.—The mice were bled from the heart under ether anæsthesia. Burroughs and Wellcome's No. 214 needles were used, the blood was collected in a glass tuberculin syringe containing 0·1 or 0·2 c.c. of sterile 4 p.c. sodium citrate and then blown into a further quantity of citrate solution in a sterile tube. As a rule about 0·5 c.c. of blood was taken from one mouse. The mortality from cardiac puncture varies somewhat with the breed of mouse, being greatest in the blacks and least in the agoutis.

The cells were washed twice in citrate and once in saline and then suspended in normal rabbit serum, 0·2 c.c. of cells being added to each c.c. of serum. This measure was adopted because it was hoped that the species-specific antibodies which rabbit sera possess might neutralize the species-specific antigens to some extent and encourage the production of other antibodies. The species-specific antibodies, however, reached an extremely high titre in many cases. Rabbits received 1 c.c. of the above suspensions in the marginal ear vein at weekly intervals. The first rabbits to be injected were bled every week and the sera tested for the presence of line-specific antibodies. Thereafter the procedure differed slightly with the line used for immunization. The number of injections was never less than 3 or more than 6. No conclusive results were obtained with "anti-black" sera. Satisfactory results were obtained with an "anti-albino" serum following the third injection, but 4 or 5 injections were advantageous. A satisfactory result with an "anti-agouti" serum was more difficult to obtain. One rabbit gave a good serum after 3 injections, but after 6 injections only species-specific antibodies could be demonstrated; serum from another rabbit gave good results after 5 injections.

All bleedings were made from the marginal ear vein 7 to 10 days after the last injection. The blood was put in the incubator for 1 to 2 hours and left on the bench overnight. The serum was inactivated for 30 minutes at 56° C. and kept in sterile bottles with 0·5 p.c. phenol. One batch of serum has been kept in this way for more than a year without deterioration. Filtration renders the serum useless and should be avoided.

Absorptions and agglutination tests with mouse erythrocytes.—Mice were bled from either the heart or the tail. In the latter case they were put in jam-jars and left in the incubator for half an hour or more. They were then anæsthetized with ether, the tail clipped and the cut end immersed in 4 p.c. citrate solution. Mice can be bled repeatedly without any detectable effect on their health. In most cases at least a week was allowed to elapse between bleedings. As a rule 5 mice will give 1 c.c. of red cells.

For absorption experiments pooled cells were used. In the earlier experiments the cells that were used for absorptions were of the same bleeding as those used in the subsequent agglutination tests, but in later experiments this was frequently not done. Absorbed sera could be kept in the ice-box for a week without deterioration even if no aseptic precautions had been observed, but in most cases not more than 24 hours elapsed between absorption and testing. Before absorption the pooled cells were washed 3 times. The first 2 washings were done with citrate solution, and the last with saline or saline and citrate solution in equal quantities. Washing with citrate reduces the hæmolysis which is very prone to occur with mouse erythrocytes. After the last washing the suspension was spun for 10 minutes at 3000 r.p.m. on an angle centrifuge. One volume of packed red cells was added to 2 volumes of serum diluted 1/10 or 1/20. (The latter dilution was usually employed.) In most cases absorptions were carried out at 37° C. for 2 to 3 hours. The effect of temperature on the specificity of reactions was mentioned in my previous paper (Gorer, 1935), and further examples are given below.

Agglutination tests were done with 1 p.c. cell suspensions in saline. Equal volumes (from ·07 to ·01 c.c.) of cell suspension and serum were incubated in precipitin tubes (about 1/4 in. internal diameter) for 2 to 3 hours. Readings were made as follows: After the condition of the deposit on the bottom of the test-tube had been observed, the supernatant fluid was sucked off in a capillary pipette and blown back on the deposit; the manner in which this breaks up varies with the degree of agglutination. The suspension is viewed in the capillary in front of a strong light after it has been mixed *gently* 3 times; it is then examined microscopically. The degree of agglutination is judged by the extent to which a film has formed on the bottom of the tube, the resistance offered to emulsification and the microscopic appearance of the resulting suspension. The symbol C. signifies complete agglutination; a.c. almost complete agglutination, etc. The least reliable of the criteria used is the appearance of the undisturbed deposit. Film formation is not invariably present at any stage below +++. The most sensitive of the macroscopic tests is the behaviour of the deposit when broken up by having the supernatant fluid blown on it. Reactions as faint as "tr." are often detectable by this test. Where tests are done in duplicate it is found that the variation is plus or minus 1°. The centrifuge technique described previously (Gorer, 1936) was used in some experiments. It is slightly more sensitive than the above method and saves time, but as the sera described in this paper were of satisfactory titre and it was necessary to incubate for at least 1 1/2 hours at 37° C. in order to obtain consistent results, it was only used occasionally to verify negative reactions.

All agglutination tests were done within 2 hours of bleeding (usually within an hour). Washing up to 5 times made no difference to agglutinability and was not always done prior to testing.

Mouse erythrocytes are very unstable, and tests left overnight and read next day are extremely unreliable.

EXPERIMENTAL RESULTS.

Tests with Normal Rabbit Sera and Immune Sera Prior to Absorption.

Normal sera from 10 rabbits were tested by direct agglutination and by absorption. In no case was it possible to differentiate between the three different mouse lines. The titres of normal sera vary between 1/50 and 1/200. The titres of the sera from 10 different rabbits were tested after immunization. The sera of the first 6 rabbits immunized were estimated one week after each injection. A titre of 1/10,000 is usual after three injections ; the highest titre observed was 1/300,000 after 6 injections. Two mice from each pure line were tested with each serum, and if there was the least indication of any difference the test was repeated. In no case was any definite differentiation obtained by this means ; the variation between the lines was never significantly greater than the variation within the lines.

Absorption Experiments.

The results obtained with anti-agouti sera.—The results of all experiments are shown in the tables in condensed form, each table representing a series of experiments. The intensity of the reactions recorded is that usually found.

Ten absorption experiments were done with 2 sera (6 with No. 2, 4 with No. 6) ; agglutination tests were done with 25 blacks, 25 agoutis and 5 albinos. The number of albinos tested is small, but later experiments with other sera confirm the results reported below. Serum No. 2 was obtained after 3 injections ; after 6 injections the reactions were less satisfactory ; serum No. 6 gave the results shown after 6 injections. One other serum gave results similar to No. 6, but was discarded because of contamination, while sera from the other 3 rabbits did not give reactions when absorbed at a dilution of 1/20, but gave feeble reactions at lower dilutions.

TABLE I.—*Absorption Experiment with* Anti-agouti *Serum No. 2. Serum Diluted* 1/20 *Prior to Absorption. Whole Experiment Performed at* 37° C.

Absorbed with cells of—	Number of times serum absorbed.	Reactions given by cells of—		
		Agoutis.	Albinos.	Blacks.
Agoutis	Once	—	—	—
Albinos	,,	—	—	—
Blacks	,,	+ to ++	+ to ++	—
,,	Twice	—	—	—

Table I shows the result of an absorption experiment with anti-agouti serum No. 2. It will be seen that a single absorption with the cells of agoutis or albinos exhausts the serum completely, but that after a single absorption with the cells of blacks, the cells of the former two groups are still agglutinated. A second absorption with the cells of blacks exhausts the serum completely. It appears that this serum contains an antibody that has a greater affinity

for the cells of agoutis and albinos than for the cells of blacks. This antibody is referred to as antibody I.

TABLE II.—*Absorption Experiment with* Anti-agouti *Serum No. 6. Serum Diluted* 1/20 *Prior to Absorption. Whole Experiment Performed at* 37° *C.*

Absorbed with cells of—	Number of times serum absorbed.	Reactions given by cells of— Agoutis.	Albinos.	Blacks.
Agoutis	Once	tr.	tr.	—
,,	Twice	—	—	—
Albinos	Once	tr.	tr.	—
,,	Twice	—	—	—
Blacks	Once	a.c. to C.	a.c. to C.	f. tr. to tr.
,,	Twice	—	—	—

C. = complete agglutination, a.c. = almost complete agglutination, tr. = trace, f. tr. = faint trace.

Table II shows the result obtained with a serum of a higher titre, anti-agouti serum No. 6. In this case a single absorption with the cells of any type still gives agglutination of the cells of that type, but the quantitative difference between the blacks and the two other pure lines is still apparent; after a single absorption with the cells of blacks, the cells of the other two groups are much more strongly agglutinated than the cells of the blacks, whilst a single absorption with the cells of the agoutis or albinos exhausts the serum *for the blacks*. A second absorption with the cells from any group exhausts the serum completely.

The cells of the agoutis and albinos are described as being of the antigenic constitution I(3) and those of blacks I(2), the figures in parentheses indicating the relative affinities for antibody I. (See Table VIII for a summary of all antigenic relationships.)

TABLE III*a*.—*The Effect of Temperature on Reactions Given by* Anti-agouti *Serum No. 2.* Absorption at 20° C. *Serum Diluted* 1/20 *Prior to Absorption.*

Absorbed once with cells of—	Temperature at which tested.	Reactions given by cells of— Agoutis.	Blacks.
Agoutis	37° C.	—	—
,,	Room temp. (20° C.)	— to tr.	—
Blacks	37° C.	— to tr.	—
,,	Room temp.	+ to +++	—

TABLE III*b*.—*Similar to above, but* Absorption at 37° C.

Agoutis	37° C.	—	—
,,	Room temp. (20° C.)	± to ++	— to ft. tr.
Blacks	37° C.	+ to ++	—
,,	Room temp. (20° C.)	C.	± to +

Tables III*a* and III*b* show the effect of temperature on the reactions given by anti-agouti serum No. 2. It appears that the amount of antibody taken up varies inversely with the temperature over the range studied, but at either temperature the difference in combining power between the two groups remains about the same. The reactions with this serum are slightly more specific at the higher temperature, and most sera do not give such predictable results at room temperature.

Results obtained with anti-albino sera.—Twenty absorption experiments have been carried out with sera of this type. Agglutination tests have been done on about 50 different animals from each of the three pure lines.

TABLE IV.—*Absorption Experiment with* Anti-albino *Serum No.* 1. Dilution Prior to Absorption 1/20. *Whole Experiment Performed at* 37° C.

Absorbed with cells of—	Number of times serum absorbed.	Reactions given by cells of—		
		Agoutis.	Albinos.	Blacks.
Agoutis	Once	—	+++ to C.	—
,,	Twice	—	++ to +++	—
Albinos	Once	—	—	—
,,	Twice	—	—	—
Blacks	Once	—	+++ to C.	—
,,	Twice	—	+++ to C.	—

Table IV shows the result of an absorption experiment with anti-albino serum No. 1. In contrast to what occurs with anti-agouti serum (*cf.* Table I), it will be seen that after a single absorption with the cells of blacks or agoutis (at a dilution of 1/20) *only* the cells of albinos are agglutinated. The antibody concerned in this reaction is referred to as antibody II.

The cells of agoutis possess some affinity for antibody II, as can be shown by means of successive absorptions. After a second absorption the cells of albinos are still agglutinated, but less strongly than after a single absorption (see Tables IV and V). Table V shows that the titre of the anti-albino serum

TABLE V.—Anti-albino *Serum No.* 1. *Diluted* 1/10 *Prior to Absorption. Demonstrating Effect of Successive Absorptions. Experiment at* 37° *C.*

Tested on cells of—	Serum absorbed *once* with cells of BLACKS. Diluted up to—				Serum absorbed *twice* with cells of BLACKS. Diluted up to—			
	1/40.	1/80.	1/160.	1/320.	1/40.	1/80.	1/160.	1/320.
Al. ♂ 149	+++	+	±	—	+++	++	±	—
Al. ♂ 137	++	+	tr.	—	++	+	ft. tr.	—
Al. ♀ 12	+++	++	+	—	+++	++	+	ft. tr.
Ag. ♂ 18	—	—	—	—	—	—	—	—
Ag. ♂ 19	—	—	—	—	—	—	—	—

Tested on cells of—	Serum absorbed *once* with cells of AGOUTIS. Diluted up to—				Serum absorbed *twice* with cells of AGOUTIS. Diluted up to—			
	1/40.	1/80.	1/160.	1/320.	1/40.	1/80.	1/160.	1/320.
Al. ♂ 149	+++	+	tr.	—	+	ft. tr.	—	—
Al. ♂ 137	++	+	tr.	—	tr.	ft. tr.	—	—
Al. ♀ 12	+++	++	+	—	+	tr.	—	—
Ag. ♂ 18	—	—	—	—	—	—	—	—
Ag. ♂ 19	—	—	—	—	—	—	—	—

Al. = Albino. Ag. = Agouti.

is slightly but significantly lowered. The amount to which the titre is lowered by absorption with agouti cells appears to vary inversely with the temperature ; one absorption at room temperature has the same effect as two absorptions at 37° C. On the other hand, the cells of blacks appear not to combine with antibody II, since the titre is not changed by a second absorption at 37° C., and is not lowered by a single absorption at room temperature.

The antigen associated with antibody II may be considered present in the cells of agoutis as II(1), in those of albinos as II(3), and absent in those of blacks (— in Table VIII).

TABLE VI.—*Demonstration of Antibody I in* Anti-albino *Serum No.* 1. *Serum Absorbed once with Cells of Blacks* Diluted 1/10. *Whole Experiment at* 37° C.

Dilution of serum subsequent to absorption.	Reactions given by cells of— Agoutis.	Albinos.
1/10	+ to ++	+++ to C.
1/20	tr to +	++ to a.c.
1/40	— to tr.	+± to +++

Control at 1/10 with cells of blacks (−).

At first it was thought that serum No. 1 did not contain antibody I, but it can be demonstrated if the dilution of the serum before absorption with the cells of blacks is reduced to 1/10 as is shown in Table VI.

TABLE VII.—*Absorption Experiment with* Anti-albino *Serum No.* 4. *Dilution Prior to Absorption* 1/20. *Whole Experiment at* 37° C.

Absorbed with cells of—	Number of times serum absorbed.	Reactions given by cells of— Agoutis.	Albinos.	Blacks.
Agoutis	Once	tr.	+++ to C.	—
,,	Twice	—	+ to +++	—
Albinos	Once	tr. to ±	tr. to +	— to ft. tr.
,,	Twice	—	—	—
Blacks	Once	C.	C.	ft. tr. to tr.
,,	Twice	—	++ to a.c.	—

N.B.—One albino ♂ gave very weak reactions with this serum. The other 19 tested fell within the range given above.

In the case of another serum, antibody I was present to a far higher titre than in serum No. 1, and it was necessary to perform two absorptions with the cells of blacks in order to demonstrate antibody II (see Table VII). One serum with which a few experiments were performed appeared only to contain antibody I.

All the albinos gave positive reactions with antibody II, but they did not all react with the same intensity, the variation at a constant dilution being as great as between C. (complete agglutination) and ++. The difference in titre was rather less than would be expected, and it seldom varied much from 1/160 with serum No. 1. Mice repeatedly tested over a period of some months gave the same type of reaction throughout. Most of the weaker reactions

were given by males, but there was considerable overlap between the sexes. No mice were used for experiments until over six weeks old, but between this age and fifteen months no differences were found attributable to age with any serum.

The variation in intensity of reactions of albinos to antibody II appears greater than the variation shown by agoutis to antibody I. In the latter case the males appear to react rather more strongly than the females.

A COMPARISON OF THE RESULTS OBTAINED WITH IMMUNE SERA AND NORMAL HUMAN GROUP A SERUM.

The results reported in a previous paper on the differentiation of mouse erythrocytes with human group A serum may be summarized as follows :

Mice selected from a heterogeneous stock (the ZS stock) gave a fairly strong reaction with unabsorbed human group A serum (titre about 1/64), whilst the agoutis and blacks gave a weak reaction (titre about 1/8). The albinos gave intermediate reactions, but it was usually possible to distinguish them from the ZS group. The results of absorption experiments were similar to those obtained with antibody I ; above a certain dilution, cells from any group would exhaust the serum completely, but with undiluted serum the three pure lines fell into a negative group and the ZS stock into a positive group. The factor detected by this method is referred to here as factor III, and may be considered as present in the agoutis, albinos and blacks as III(2) and in the ZS animals as III(3).

Some ZS animals gave fairly strong reactions with antibody II, but these were weaker than those usually obtained with the cells of albinos. Other ZS animals gave very weak reactions on direct agglutination (f. tr. or —), but were shown to have some affinity for the antibody by means of absorption experiments. A single absorption of an anti-albino serum by such ZS animals lowers the titre for albinos, whilst a second absorption exhausts the serum completely. ZS animals giving this kind of reaction are designated as II(2) in Table VIII. Most ZS animals appear to possess the antigen I (3). Unfortunately experiments with this group could not be carried as far as could be wished, owing to the outbreak of a respiratory epidemic which necessitated the destruction of the stock.

Below is a table summarizing the antigenic relationships of the stocks so far studied.

TABLE VIII.—*Antigenic Constitution of Mouse Erythrocytes from Strains so far Studied.**

Strain of Mouse.	Antigenic factors.		
Agouti	I(3)	II(1)	III(2)
Albino	I(3)	II(3)	III(2)
Black	I(2)	—	III(2)
Selected from ZS	I(3)	II(2)	III(3)

Roman numerals indicate antigenic factors. Arabic numerals in parentheses indicate relative avidity for corresponding antibodies ; (3) signifying maximum avidity. — = antigen absent.

* Recently a fourth antigen has been demonstrated with some new anti-black sera. Blacks have the antigen IV(3), agoutis IV(1) ; there are at present insufficient albinos available for a detailed investigation.

DISCUSSION: THE SIGNIFICANCE OF QUANTITATIVE DIFFERENCES.

Since cross reactions appear to occur frequently with mouse corpuscles, it will be as well to consider their significance. Such reactions could be given under the following conditions:

1. They might occur if two cells have different quantities of a definite chemical substance. Conclusive evidence of a difference of this kind is difficult to obtain in the absence of accurate chemical analysis. It seems probable that the lower titre given by the human antigens M and N when heterozygous is due to a difference of this kind (Wiener, Zinsher and Selkowe, 1934).

2. Two or more molecules may differ in the proportions of certain determinant groups. The sub-groups of the human group A (A_1 and A_2) may differ in this manner. Thomsen *et al.* (1930) found that the former type had a greater avidity for the normal iso-agglutinin than the latter, but A_2 corpuscles react more strongly with certain other sera (Landsteiner and Levine, 1926, 1929 and 1931; Landsteiner and Witt, 1926; and Schiff, 1934). It is found that sera reacting specifically with A_2 usually show a similar type of reaction with cells of group O. Genetically A_1 is dominant to A_2, which is dominant to O; it is possible here that successive mutations in the same gene cause a molecule to be modified to a varying extent.

3. Investigations with synthetic antigens have shown that closely related chemical substances may give rise to cross-reactions. (See Landsteiner, 1933, and Marrack, 1934, for discussions of this subject.)

4. Landsteiner and van der Scheer (1934) reported that artificial antigens containing aliphatic radicals with long carbon chains showed less specificity than those with short carbon chains.

We cannot yet place the antigenic differences existing between mouse erythrocytes into one of the above categories; at present it is convenient to describe them as quantitative differences.

Should the genetic basis of the antigenic differences so far described be simple, we shall have a useful additional method for extending our knowledge of the genetics of mice; should it be found that certain of the differences are due to multiple factors, it may be found possible to throw some light on the nature and origin of intraspecific differences which appear (at least with closely related species) to be due to multiple genetic differences.

SUMMARY AND CONCLUSIONS.

1. Rabbits were immunized with the erythrocytes of mice from three lines that had been brother-sister inbred for over 25 generations. Such strains may be considered genetically and antigenically uniform. The three strains used are referred to as the agoutis, albinos and blacks.

2. Sera from normal rabbits and *unabsorbed* sera from immunized rabbits react similarly on the erythrocytes from all three pure lines.

3. Absorption tests with anti-agouti sera showed that such sera may contain an antibody acting more strongly on the cells of the agoutis and albinos than on the cells of blacks. This antibody is referred to as antibody I. The cells

of agoutis and albinos are described as being antigenically I(3), and those of the blacks as being I(2). The figures in parentheses indicate the relative affinities for antibody I.

4. Anti-albino sera may contain antibody I and a different antibody (antibody II). The cells of blacks do not react at all with antibody II, those of agoutis react very feebly, whilst the cells of albinos react strongly. Mice selected from a heterogeneous stock (the ZS stock) gave reactions intermediate in strength between those given by the agoutis and albinos.

5. A comparison is made between the results obtained with immune sera and those obtained with normal human group A serum. A factor detectable by the latter serum is here referred to as antigen III.

The antigenic relationships between all the groups studied are summarized in tabular form.

6. A discussion of the significance of "quantitative" differences is given in the text. It is concluded that such differences are attributable to four possible causes, which cannot yet be distinguished from one another. The term "quantitative" should be regarded as a convenient label signifying the fact that different cells react with different intensities with the same antibody.

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THE SIZES OF THE VIRUSES OF HUMAN AND SWINE INFLUENZA AS DETERMINED BY ULTRAFILTRATION.

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THE viruses of human influenza (W.S. strain ; Smith, Andrewes and Laidlaw, 1933) and of swine influenza (Shope, 1931), as contained in broth suspensions of lung tissue from infected mice, have been subjected to differential ultrafiltration through graded collodion (gradocol) membranes with a view to estimating the sizes of the infective agents.

METHODS.

The membranes used and the filtration set-up were precisely as described in previous studies from this laboratory [*cf.* Elford (1931), Barnard and Elford (1931), Elford and Andrewes (1932)]. In all experiments filtration was conducted under positive pressures of nitrogen.

Filtrates to be tested were inoculated intranasally into young mice under light ether anæsthesia ; each mouse received approximately 0·05 c.c. Each dilution of filtrate was tested on a group of three mice. All mice surviving were killed 5 days after inoculation and their lungs examined for the typical lesions (*cf.* Andrewes, Laidlaw and Smith, 1934). The highest dilution of filtrate causing typical lesions in one or all of the mice in a group was taken as the virus titre.

Stock suspension of virus was prepared as follows: Six or more mice were inoculated intranasally with virus, usually virus filtrate, and killed after 2 or 3 days. Their lungs were ground with powdered Pyrex glass, and made up to a 5 p.c. suspension with a mixture of broth and saline in equal parts. This was centrifuged for 10 minutes, clarified by filtration through paper pulp and asbestos pulp or asbestos only, and then filtered through a coarse membrane (average pore diameter 0·65 to 0·9μ). This stock filtrate was usually active in a dilution of $1 : 10^4$ or $1 : 10^5$ in the case of the human virus, $1 : 10^3$ or $1 : 10^4$ with the porcine strain.

RESULTS.

Human Influenza Virus.

The experiments in which stock filtrate was filtered through membranes of progressively lower porosities are summarized in Table I.

It is clear that a membrane of 0·14μ A.P.D. completely retains the virus, while one of 0·18μ A.P.D. allows a very little to pass. As has been noted with other viruses, some virus begins to be retained when one reaches a membrane with a pore diameter about twice that of the limiting size ; this indicates that the particles are of fairly uniform size.

TABLE I.—*Filtration of Human Influenza Virus.*

Titre of stock virus filtrate.	Average pore diameter of membrane (in μ).	Titre of final filtrate.	Percentage of virus passing filter.
10^4	0·40	10^4	100
10^4	0·25	10^3	10
10^4	0·22	Undiluted	0·01
10^5	0·22	10	0·01
10^4	0·18	Undiluted	0·01
10^4	0·18	Negative	..
10^4	0·18	Undiluted	0·01
10^5	0·18	,,	0·001
10^3	0·14	Negative	..
10^4	0·14	,,	..
10^4	0·14	,,	..

In Tables I and II the titre of the virus is the highest dilution of filtrate which will infect a mouse in an 0·05 c.c. dose.

Swine Influenza Virus.

Similar experiments made with swine influenza virus yielded the results contained in Table II.

TABLE II.—*Filtration of Swine Influenza Virus.*

Titre of stock virus filtrate.	Average pore diameter of membrane (in μ).	Titre of final filtrate.	Percentage of virus passing filter.
10^3	0·42	10^3	100
10^3	0·34	10	1
10^3	0·34	100	10
10^4	0·26	100	1
10^3	0·22	Negative	..
10^3	0·22	,,	..
10^3	0·22	Undiluted	0·1
10^4	0·18	,,	0·01
10^3	0·18	Negative	..
10^3	0·18	Undiluted	0·1
10^3	0·16	Negative	..
10^3	0·15	,,	..
10^3	0·15	,,	..
10^3	0·15	,,	..

The results are almost identical with those obtained for the human virus.

CONCLUSION.

The end-point for each of these strains of influenza virus may be taken as 0·16μ, from which, on the basis of the relationship between limiting porosity and particle size established by Elford (1933), we deduce the probable size of the virus particles to be 80–120mμ. The virus of influenza is thus seen to be

somewhat smaller than the viruses of vaccinia and herpes (125–175mμ. and 100–150mμ. respectively).

The results confirm the earlier conclusion that the diameter of the viruses is less than 0·3μ (Andrewes, Laidlaw and Smith, 1934). Further, by showing that they are, in fact, considerably smaller than that figure, they appear to render it extremely improbable that the viruses can represent a small form of *Hæmophilus influenzæ*, or of any other bacterium which has been suggested as a cause of influenza.

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COMPARATIVE STUDIES OF THE LOUSE-BORNE (EPIDEMIC) AND FLEA-BORNE (MURINE) TYPHUS VIRUSES.

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THE last few years have witnessed a considerable advance in our knowledge of the ætiology and epidemiology of typhus-like diseases. The work of Maxcy (1929) on the epidemiology of a typhus-like disease in the southern United States suggested that there was a carrier other than the louse, and led him to suspect the rat as an animal reservoir of the virus. This work, following closely that of Mooser (1928), who established the difference in the scrotal reaction caused by the Mexican and European *Rickettsia* respectively, indicated the existence of two different types of this disease. Finally the discovery by Dyer and his associates (1931) of the virus in rat fleas in a typhus focus in the United States, and by Mooser, Castaneda and Zinsser (1931), in the brain of wild rats in Mexico City, brought forth conclusive evidence in support of Maxcy's assumption, and cleared up the epidemiology of the Mexican and American variety of typhus. Subsequently Lepine (1932) showed that Mediterranean typhus was also rat-borne, and that that virus produced the same reaction in guinea-pigs as did the American and Mexican types. Simultaneous studies in various parts of the world have proven the wide distribution

of typhus-like diseases and the existence of several distinct forms due presumably to different species of *Rickettsia*.

The problem which has concerned us as well as a number of other investigators is the relation of the flea-borne rat virus to the louse-borne human virus. It seems reasonable to assume that the rat constitutes a natural animal reservoir of a virus closely related to that producing the European type of the disease, and this assumption is strengthened by the close immunological response of the two forms of the disease. On the other hand, evidence is accumulating of their diverse pathogenicity for rats, mice and guinea-pigs, while cross-protection experiments suggests also certain antigenic differences.

As regards pathogenicity, the differences are striking and, in our experience, constant. The special affinity of the rat strain for the tunica of guinea-pigs has been repeatedly demonstrated. Although we have been able to confirm Pinkerton's findings of *Rickettsia* in the tunica of guinea-pigs infected with the Breinl strain, the quantitative difference was striking; in the louse-borne virus it is possible to find them regularly only in cultures of the mesorchium, and not in the tunica itself. Moreover, in our experience, the special affinity of the rat strain for the scrotum is quite constant. Even after 26 brain-to-brain passages in mice, the strain produced typical scrotal lesions in guinea-pigs. Another constant difference is the pathogenicity of the two varieties for mice and rats. The rat strain is pathogenic for rats, while the European strain is not. Repeated attempts made by us to establish the latter in rats have failed; after the third or fourth passage the strain is lost. On the other hand, the rat strain is easily maintained in white rats, and we have also carried it in mice through 31 passages.

Antigenically the two strains seem closely related. Both produce agglutinins for HX19, and Lépine (1933) has shown that animals recovering from an infection with one type are immune to an infection with the other. At the same time Zinsser and Castaneda (1933) have found that guinea-pigs immunized with their rat strain vaccine were not protected against an infection with the European virus.

Although both strains are readily cultivated in tissue cultures, it is not possible to make an antigenic analysis of the organisms because of the relatively small amount of antigen obtained. We undertook, therefore, a study of the immunological relation *in vivo*. It occurred to us that cross-protection tests and, more particularly, cross-immunization with vaccines prepared from cultures of the two varieties might yield information on this point.

CROSS-IMMUNITY TESTS.

Immunity after an Infection.

In a series of guinea-pigs we tested the immunity of animals which had recovered from one infection against a reinfection with the other type. Fifty-seven animals which had recovered from an infection with the rat strain were infected with the European virus (Breinl strain), and, conversely, 17 guinea-pigs which had recovered from an infection with the European virus were reinfected with the rat strain. The reinfections were carried out one to two months after recovery.

The results correspond with those reported by Lépine. Of the groups reinfected with the rat strain, 2 developed an aborted and 1 an attenuated infection. The others showed neither rise in temperature nor, in case of males, any suggestion of scrotal swelling. Of the group reinfected with the European type, 3 out of 17 developed mild infections of 2–3 days' duration ; the others were immune. It may be concluded, therefore, that an infection with one type produced a solid immunity, adequate also against an infection with the other type.

Cross-Immunity after Immunization with Vaccine.

In another series of animals we tested the immunity produced by vaccination against the homologous and heterologous strains. The procedure was to immunize a group of animals with a given vaccine, and then infect half of these animals with the European and half with the rat strain. The vaccines were prepared from tunica tissue cultures of *Rickettsia* in a manner described in a previous publication (Kligler and Aschner, 1934). For purposes of immunization the guinea-pigs were given three injections of the formolized vaccine at intervals of 3 days. The dose was either 0·5 or 1·0 c.c. of vaccine. In all experiments animals of medium weight (300–400 g.) were used. As a rule the infecting virus was given 7 to 10 days after the last injection of the vaccine. Controls were used in every case so that the infectivity of the virus was assured. The infecting virus was taken from guinea-pigs at the height of the infection. In the European strain 0·5 to 1·0 c.c. of a 1 : 100 brain emulsion was used ; in the rat virus 1·0 c.c. of 1 : 30 emulsion of testicle and spleen was injected. The results are summarized in Tables I and II. Table I gives the results of vaccination with the rat virus vaccine and Table II with the European vaccine.

TABLE I.—*Immunization with Rat Virus Cultures and Infection with Rat and European Virus Respectively.*

Number of animals.	Immunizing doses.	Date of last injection.	Date of test infection.	European virus.		Rat virus.	
				Number of animals.	Results.	Number of animals.	Results.
2	3 × 1 c.c.	7.v	14.v	1	1 negative	1	negative
4	3 × ½ c.c.	„	17.v	2	2 „	2	2 „
9	3 × ¼ c.c.	17.vi	25.vi	4	4 positive	5	2 aborted
9	3 × ½ c.c.	„	„	4	1 aborted 3 positive	5	5 negative
9	3 × 1 c.c.	„	„	4	4 „	5	5 „
8	3 × 1 c.c.	15.vii	26.vii	4	1 „ 2 aborted 1 negative	4	4 „
5	3 × 1 c.c. i.p.	24.x	4.xi	5	3 positive 1 aborted 1 negative		
5	3 × 1 c.c. i.c.	„	„	5	2 positive 2 negative 1 secondary infection		

TABLE II.—*Immunization with European Virus Vaccine and Infection with Rat and European Virus Respectively.*

Number of animals.	Immunizing doses.	Date of last injection.	Date of infection.	European virus.		Rat virus.	
				Number of animals.	Results.	Number of animals.	Results.
5	3 × 1 c.c.	14.xi	22.xi	2	2 negative	3	1 positive 1 negative 1 aborted
6	3 × ½ c.c.	„	„	3	2 „ 1 aborted	3	2 negative 1 positive
10	3 × 1 c.c.	24.xii	2.i	5	5 negative	5	1 aborted 3 negative 1 positive

Note.—An animal is considered immune when it fails to react to an infection with 1 c.c. of 1 : 100 brain emulsion, in the case of European virus, or ½ c.c. of 1 : 30 suspension of testicles and spleen, in the case of rat virus.

An infection is considered aborted when the temperature does not exceed 39·5° C., and more particularly when its duration is only 1–2 days instead of the usual 5–6-day period.

It is apparent from the results that, when tested by this procedure, the two strains show antigenic dissimilarities not noted when animals which have recovered from an infection are reinfected with the heterologous virus. Thus, of 42 animals immunized with the rat virus, the 17 infected with the homologous strain proved fully immune, while of 25 infected with the human virus, 13 developed a typical infection, 4 an aborted infection, and only 7 (28 p.c.) were definitely immune. In the series of 21 animals immunized with vaccine prepared from human virus, the 10 infected with the homologous strain proved immune (9 fully, 1 partially), while of the 11 infected with the rat strain, 6 proved fully immune, 2 were partially immune and 3 were not immune.

These results correspond with those reported by Zinsser and Castaneda (1933), who found that, among 6 animals vaccinated with the murine vaccine and subsequently reinfected with the European virus, only 1 was immune, 3 developed aborted infections, and 2 typical infections.

They are in striking contrast with results obtained on reinfecting animals with a heterologous strain after recovery from an infection. Evidently there is a difference between the nature of the immunity produced by an infection and that produced by vaccination. Whether this difference is qualitative or quantitative in character still remains to be determined. Absorption experiments of immune sera with tissue culture suspensions have thus far failed to yield clear results. It would appear that the two strains are antigenically dissimilar, either in the quantitative relation of their antigenic elements, or in the presence in one of an antigenic element absent in the other.

The results reported in this paper confirm our previous findings (1934) that it is possible to immunize animals with killed tissue culture vaccine against an infection with the homologous strain. They show further that there is some antigenic difference between the two strains sufficient to render it impracticable to use the vaccine of the murine strain for immunization against the European virus.

THE MAINTENANCE OF THE TWO TYPHUS VIRUSES IN MICE BY BRAIN-TO-BRAIN PASSAGE.

The discovery that the rat is a natural host of a typhus virus which is closely related to the human louse-borne virus has raised two important questions. One concerns the origin of the human virus, and the other the identity of the louse-borne and flea-borne *Rickettsia*. While it is generally conceded that at some time or other the human virus may have been derived from the rat strain, there is a division of opinion as to that occurring at present. The differences in the pathogenicity of the human and rat strain respectively for white rats has been demonstrated by various investigators. Nicolle and Laigret (1933), Laigret and Jadin (1933) and Savoor and Velasco (1934) have shown that this also holds true for white mice.

The object of the present experiments was to ascertain (1) whether it is possible to maintain the two varieties in mice by brain-to-brain passage ; (2) whether a long series of such passages would in any way alter the character of the strains used.

Experimental.

Four experiments were made with the human strain. Mice were inoculated with 0·02 c.c. of a 10 p.c. suspension of infected guinea-pig brain, and passages of an emulsion of brain and spleen made at various intervals intracerebrally to mice and intraperitoneally to guinea-pigs.

In the first experiment the mice were inoculated with a brain emulsion. The mouse brain was infective for the guinea-pig 17 days after the injection, but was not infective after the second passage.

In the second experiment mice were injected intracerebrally with brain emulsion from a guinea-pig. Twelve days later a brain passage was made into another set of mice and an intraperitoneal injection into a guinea-pig. The guinea-pig developed an atypical infection. After 10 days a second mouse-to-mouse passage was made—a brain and spleen emulsion being used ; 10 days later a third passage was made into mice, and at the same time a guinea-pig was injected, with positive results. Two more passages were made at intervals of 12 days, and this time the guinea-pig injection was negative. The strain was maintained, therefore, in the mice during three brain passages.

In the third experiment the first passage from the infected mice was made 10 days after the injection. The brain emulsion was infective for guinea-pigs Twelve days later a second passage was made, and 13 days later a third passage. Guinea-pigs injected with the third passage brain did not react. The virus was, therefore, lost by the third passage.

In the fourth experiment the virus was lost after the first mouse passage.

These results confirm, therefore, those reported by Laigret and Jadin (1933), namely that the human virus cannot be maintained in mice by brain-to-brain passage for more than 1 to 3 passages. We had no difficulty in establishing the rat virus in mice by brain-to-brain passage. We have carried the strain through 31 mouse passages. The first 18 passages were made on the sixth to eighth day after the injection. Subsequent passages were made on the eighth to tenth day. The strain has been maintained in mice from 13. ii to

20. ix—a period of seven months. At intervals passages were made into male guinea-pigs in order to test the virulence of the strain, and more particularly its scrotal affinity. At no time have we noted any change in virulence or modification of the scrotal involvement. Guinea-pigs injected with the mouse brain emulsion of the 31st passage reacted in a typical manner with scrotal swelling and the presence of *Rickettsia* in the tunica.

Reaction of Mice.

As noted by other workers, the mice develop an inapparent infection. No change can be noted in the outward appearance of the mice. The fur remains smooth, the mice remain active, and in general there is no external evidence that the animals are passing through an infection. In looking for an indicator of infection corresponding in some way to the temperature index in guinea-pigs we undertook a series of blood-counts. Blood was taken from the tail and daily counts made in the morning before feeding. It was found that the normal white-cell count ranged between 7500 and 12,000 per c.mm., and that the lymphocytes predominated, the proportion usually being: L. 70–75 p.c. and P. 25–30 p.c. In mice injected intracerebrally with *Rickettsia* the count fluctuated around this figure during 3–4 days after the inoculation, and then

TABLE III.—*White Cell-count in Mice Injected Intracerebrally with a Murine Strain of* Rickettsia.

INFECTED: WHITE CELL-COUNT (NUMBERS REPRESENT THOUSANDS).

	Days after injection.									
	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
1	..	..	9·9	..	11·0	8·4	..	7·2	6·0	..
2	..	..	13·8	8·1	9·0	9·5	9·7	10·0	9·8	9·2
3	11·5	11·2	9·8	10·5	9·6	9·7	11·0	8·0	10·6	9·6
4	*25·3*	*21·8*	*17·5*	*13·1*	*17·7*	*15·7*	*14·8*	11·2	*16·0*	11·4
5	*22·5*	*27·5*	*19·3*	*16·5*	*17·0*	*16·0*	*16·2*	*12·2*	*18·6*	12·4
6	*18·4*	*20·2*	*17·3*	*18·2**	*15·9*	*13·9*	*15·2*	*15·0*	*19·8*	*14·9*
7	*17·2*	*18·5**	*16·2**	..	9·5	9·0	15·2*	*17·0*	17·0*	*14·4*
8	*16·7*	..	..	..	9·8	7·8	..	8·0	..	*13·5*
9	*16·7*	..	..	..	..	..	..	7·6	..	12·7
10	13·3	..	..	..	..	..	..	..	..	..

CONTROLS (NORMAL BRAIN INJECTION).

	Days after injection.						
	1.	2.	3.	4.	5.	6.	7.
1	9·5	8·5	8·4	8·5	10·8	8·0	12·0
2	9·1	8·7	9·7	9·0	10·6	10·0	10·8
3	10·8	7·8	9·6	12·8	10·4	11·5	11·4
4	9·2	10·3	9·0	10·0	9·9	11·0	10·4
5	10·8	11·2	11·8	10·0	9·6	8·2	7·2
6	10·9	9·2	12·0	9·0	9·2	7·0	8·2
7	11·7	11·6	12·4	9·0	9·8	7·2	7·4
8	9·9	11·3	12·5	..	..	7·2	7·6
9	11·2	11·4	..	..	..	8·0	9·8

* Killed for passage. Guinea-pigs developed typical infection with scrotal swelling.

rose either slowly or sharply; as a rule the rise in the white cell-count was accompanied by a shift in the L/P ratio. This state persisted during 3–5 days and the count then returned to normal. Mice inoculated with a normal mouse brain suspension did not show a similar rise in the white-cell count. Table III gives a number of typical counts of infected and control mice. The regularity of the leucocytosis in infected mice after a fairly constant period of incubation suggests that the rise in white cell-count is a response to the infection, and may serve as a useful index of the infection in mice.

In the course of our work we have had occasion to observe a paratyphoid epidemic in our mice, and have found that in this case, too, there is a leucocytosis which is indicative of the infection. This proved particularly useful in weeding out infected animals. Often animals were found normal the first day, but gave a sharp increase the next. Such animals, when killed and cultured, invariably turned out to be infected. In some cases all the organs were positive; in others the infection was only at the beginning, the spleen and mesentery being involved. But in all cases a high white-cell count was associated with an infection.

SUMMARY.

(1) Guinea-pigs which have recovered from a louse-borne (European) variety of typhus (Breinl strain) are immune to an infection with the rat strain and *vice versa*. In the latter case the immunity is not quite so effective as in the former.

(2) Guinea-pigs immunized with formolized tissue culture vaccine of either variety develop a solid immunity against the homologous strain, but are not, as a rule, protected against the heterologous strain.

(3) The human strain of typhus could not be maintained in mice by brain-to-brain passage for more than one to three passages.

(4) The rat strain could be easily maintained in mice by brain-to-brain passage. Thirty-one cerebral passages in mice during a period of 7 months did not in any way alter the virulence of the virus for guinea-pigs or its affinity for the tunica vaginalis.

(5) The infection in the mouse is inapparent. It was found, however, that the mouse reacts by a leucocytosis which appears after an incubation period of 3–4 days, and persists for 3–5 days. At the same time there is a relative increase in the polymorphonuclear leucocytes. It is suggested that the increased leucocyte count may serve as an index in an inapparent infection in mice corresponding to the rise in temperature noted in other animals.

(6) The experiments on cross-immunization, as well as those in mice, indicate that typical louse-borne and flea-borne strains of *Rickettsia* can be differentiated both antigenically and biologically. The accumulated evidence supports Nicolle's view that the two types, though probably having a common origin, are at present distinct.

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PRODUCTION OF THE CBA STRAIN OF INBRED MICE: LONG LIFE ASSOCIATED WITH LOW TUMOUR INCIDENCE.

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UP to two years ago spontaneous tumours in mice belonging to the inbred CBA strain were extremely rare. The strain had been produced primarily by a brother-to-sister mating since 1920. On account of this rarity of spontaneous tumours, a selection experiment toward longevity was set up. This procedure was followed since it was obvious that for a strain of mice to be highly resistant to spontaneous tumours, the mice ought to live well beyond the age variability of the common types of spontaneous tumours in that species of animals. Since carcinoma of the female mammary gland and carcinoma of the male lung are the commonest types of tumours found in mice, and since these manifest themselves on an average of 10–12 and 14–16 months respectively, it became highly desirable to have resistant mice live considerably beyond this age-period.

In the present selection experiment only the descendants of that mouse in each generation which lived longest were continued. By 1930 an inbred strain had been obtained, the individuals of which, in direct descent for ten generations, averaged more than 24 months of life without any of them showing signs of tumours. Sporadically, however, in the collateral lines, several types of tumours had been discovered. The direct descendants of these collateral mice, however, were always discarded.

Between December 15th, 1933, and June 26th, 1934, 71 females of the CBA strain that had been used for breeders were placed in reserve, free from further production of young, on a mixed " oatmeal diet " consisting of Quaker rolled oats, Baugh & Sons meat scrap, Klim powdered whole milk and salt.

The ages at which these mice had produced their first litters of young

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before being placed in reserve are given in Chart 1. This is a fair sample of beginning functional sexual maturity (minus 19–20 days for the time of first gestation), since the females had been mated to males at weaning age (25–40 days of life).

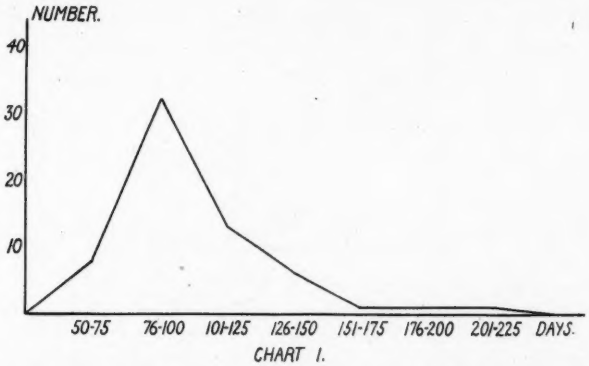

CHART 1.—This chart shows the age at which the female mice of the CBA strain had produced their first litters. The average age for first litters was 99·0 days of life.

The CBA females had been born at different times throughout the year. Chart 2 presents the data on first litters, after the mice had been grouped according to the month of the year in which they had been born. Those mice which had been born during the warmer months of the year (particularly July

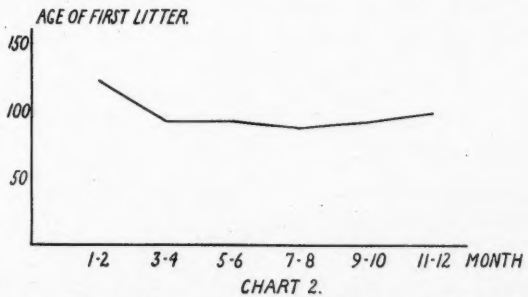

CHART 2.—This chart shows the age of first litters for female mice of the CBA strain, after the females were divided into groups depending upon the month of the year in which they had been born.

and August) produced their first litters earlier than those mice which had been born during the colder months (particularly in January and February) (88 days of life compared to 122 days).

Chart 3 gives data on (1) the age at which the mice were placed in reserve —broken or dash line; (2) the age at death of 67 of the 71 mice which died of

some other cause than tumours—solid line; (3) the ages for the occurrence of two cases of spontaneous carcinoma of the mammary gland (mouse 96598 at 732 days, and mouse 90803 at 557 days)—dot and dash line; and (4) the

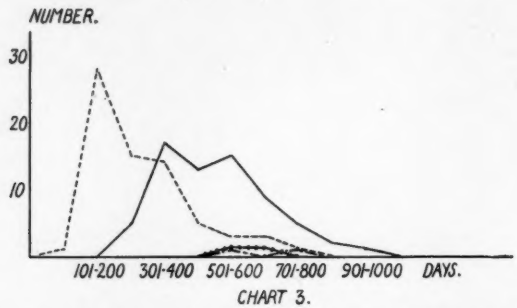

CHART 3.

CHART 3.—Chart 3 shows data on (1) the age at which the mice were separated from further reproduction and placed on an "oatmeal diet" (dash line), (2) the age at death of the 67 mice which died of other causes than cancer (solid line), (3) the age of the 2 mice which developed spontaneous carcinoma of the mammary gland (dot and dash line), and (4) the age for the 2 mice which developed spontaneous hepatoma or adenoma of the liver (ball and solid line).

ages for the two mice which developed spontaneous hepatoma or adenoma of the liver (mouse 92452 at 602 days and mouse 91141 at 581 days)—ball and solid line.

DISCUSSION.

For the past fifteen years, ten to twelve stocks of mice have been rigidly inbred by brother-to-sister matings. Some of these strains were derived from common sources; others are entirely unrelated to each other. Very few hybridization experiments have been performed. The purpose in this procedure of carrying a large colony of mice over a period of years is the comparative study of the origin of spontaneous tumours under the same environmental conditions. In other words, all strains of mice have been subjected to the same environmental conditions. The one great variable was the intrinsic or genetic determiners of physiological variation. Although the colony has been moved several times during this regime, the mice of the various strains have always been kept simultaneously in the same room.

It is a significant fact that specific types of tumours do arise in the different inbred strains. Examples of this are (1) carcinoma of the mammary gland in the females of many of the stocks, (2) primary carcinoma of the lung in both males and females of the A strain, and (3) visceral tumours, such as adenoma of the liver, uterine and ovarian carcinoma, in mice of the CBA strain. A segregation of specific types of tumours in specific organs would indicate that we were dealing with specific or qualitative determiners transmitted through the germ-plasm. On the other hand, the graded series of age-incidence of primary carcinoma of the female mammary gland cannot be so completely explained. Beginning at eight months, which is the average age-incidence of

spontaneous tumours for females of the C_3H strain (Strong, 1935), through 10 to 11 months, for females of the D inbred strain, 12 months for females of the A strain (Strong, 1936), 14 to 16 months for females of the $C_{12}I$ strain, 18 to 20 months for females of the C strain, and 24 to 26 months for females of the CHI strain, there is a progressive series without an apparent break. Beyond this, however, cancer of the female mammary gland occurs sporadically in mice of such a strain as the CBA, which has been produced by a continued selection toward longevity. Thence the series grades off into the more resistant strains, until finally in some strains of mice carcinoma of the female mammary gland has never been encountered. Observations such as the above have convinced us that we are dealing, not with qualitative, but with quantitative differences. The mechanism which apparently controls resistance and susceptibility to spontaneous tumours in mice is a delicate or sensitive one. This difference is perhaps the slightest between females of the A strain, which are highly susceptible to development of carcinoma of the mammary gland, and the CBA females where a carcinoma of the same gland is encountered rarely and at very late periods of life.

Inbred strains of mice are now being employed in many parts of the world for cancer research. The coal-tar derivatives, chemically pure sterols and hormones are being extensively used. The present study is published at this time, primarily for the purpose of emphasizing the fact that the intrinsic determiners within the organism cannot be ignored. We do not yet know what the normal individual will do without the influence of injected chemical agents. Until we do know what highly inbred mice will do, we cannot hope to analyse hybrid generations of mice or mice subjected to additional unknown variants.

CONCLUSIONS.

1. The female mice of the CBA inbred strain, up to 18 months of life, are relatively resistant to spontaneous carcinoma of the mammary gland. This applies to females that have been used for a moderate degree of breeding and kept on the " oatmeal diet " used in this experiment.

2. Visceral tumours, including adenoma of the liver, may be expected in female mice of the CBA strain.

Note.—Visceral tumours, exclusive of adenoma of the liver, but including uterine and ovarian neoplasms, also arise spontaneously in females of the CBA strain, but this conclusion is not based on the present study.

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EXPERIMENTS ON THE CHEMICAL BEHAVIOUR OF POTATO VIRUS "X".

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ESTIMATES of the size of virus particles have been made by a number of different methods, and there is substantial agreement that few have a radius of less than 10mμ. A spherical particle of this radius and with a specific gravity of 1·2 would weigh 5×10^{-18} g., or as much as 3×10^6 hydrogen atoms. Since about two-thirds of this weight is probably water, the solids in such a virus can be assumed to have a weight similar to that of a molecule of molecular weight one million. Such a particle is comparable with the largest proteins, *e. g.* hæmocyanin (Svedberg, 1932), but it could contain many molecules with weights in the more usual range (35,000 to 200,000) for large molecules. It is therefore reasonable, until good evidence for the chemical individuality of a virus particle is obtained, to assume that a virus is chemically complex. There is evidence from filtration experiments that potato virus "X" and certain animal viruses have radii much greater than 10mμ., and are therefore presumably correspondingly heavier (Smith, 1933). The experiments recorded in this paper suggest that protein is an essential part of virus "X", but there is no evidence that other equally important substances may not also be present.

The study of the effects of enzymes on plant viruses has so far been restricted to viruses of the tobacco mosaic type, but even so the published results are conflicting. Lojkin and Vinson (1931) found that tobacco mosaic virus in purified suspensions was inactivated by trypsin, but that this enzyme had no effect on crude infectious sap. Matsumoto and Somazawa (1934) repeated and confirmed this, but they found that even when infectivity was completely destroyed by trypsin the antigenic properties of the virus were not affected. Caldwell (1933, 1935), also, found that trypsin inactivated tobacco mosaic virus but that the activity could be restored by heating. Stanley (1934*a*) confirmed this, and put forward the view that the action of trypsin is on the host-plant and not on the virus. The inactivation occurred as soon as the virus and enzyme were mixed and did not increase on incubation.

Lojkin and Vinson (1931) and Caldwell (1933) found that pepsin had no effect on tobacco mosaic virus, whilst Stanley (1934*b*) states that it slowly inactivated the virus. Lojkin and Vinson and Caldwell found that papain reduced the infectivity, but were uncertain that the effect was due to proteolysis.

MATERIALS AND METHODS.

The experiments described below were made with purified suspensions of the S strain of potato virus " X ", which produces necrotic local lesions and systemic symptoms of necrotic spots and rings in infected tobacco plants (var. White Burley).

In any experiment which involved the addition of either acid or alkali the virus suspensions were carefully neutralized before testing. The experimental fluids were tested both for their infectivity and for their flocculating power with the sera of rabbits immunized against virus " X ". The inoculations to plants were made with ground-glass spatulæ, and the suspensions were rubbed as evenly as possible over the leaves. The flocculation experiments were carried out in 7 mm. thick-walled glass tubes, 0·9 c.c. of antigen at a constant dilution being added to a series of tubes each containing 0·9 c.c. of antiserum at varying dilutions. The tubes were immediately placed in a water-bath at 50° C. in such a manner that convection currents kept the mixtures continuously agitated, and they could be observed without being moved. The optimal tube was taken as that which first showed any flocculation, and the concentration of serum in this tube was used as an indication of the strength of the antigen. This has previously been shown to be closely related to the virus content as measured by the capacity to produce local lesions (Bawden, 1935).

The purified virus suspensions were prepared in two ways. At first the method described by MacClement (1934) in which the virus is precipitated by CO_2 was used. As this is laborious and entails centrifuging large volumes of fluid, in the later work the following method, which has proved equally effective, was substituted :

Young leaves from infected tobacco plants are frozen overnight at −5° C., put in a muslin bag and the sap expressed from them by means of a hand-press. The sap is diluted 1 in 4 with ice-cold water and brought to pH 5 by the addition of $N/10$ HCl. After centrifuging, the yellowish-brown supernatant fluid is further acidified to pH 4. 100 c.c. of expressed undiluted sap require about 15 c.c. of acid to bring it to pH 5, and a further 30 c.c. to bring it to pH 4. It will be shown later that centrifuging at pH 4 precipitates all the virus from purified suspensions, but this is not so with crude plant extracts. The supernatant fluid obtained at pH 4 during the preparation still contains some of the virus. This can be precipitated by the addition of an equal volume of alcohol, and after resuspension in water can then be precipitated at pH 4. The precipitates at pH 4 are resuspended in water and dilute NaOH added to bring the pH to 7, when the suspensions are again centrifuged. The supernatant fluids are the purified virus suspensions, and are usually slightly opalescent. The amount of opalescence varies with different preparations, and by centrifuging at high speeds water-clear suspensions can be obtained without appreciably affecting the virus content. Water-clear preparations from 100 c.c. of expressed undiluted sap contain about 10 to 15 mg. of non-dialysable material. Suspensions containing 1 mg. per c.c. of this non-dialysable material are pale brown in colour, and react with antiviral sera at a dilution of 1 in 640.

Some analyses have been made on these solutions, although it is probable, for a number of reasons, that the virus is not even the principal solid constituent. In the first place a product with very similar chemical properties can be isolated in only slightly smaller yield from the sap of healthy plants by the method used for the purification of the virus; this product is, of course, non-infectious and gives no precipitate with antiviral sera. The relatively low end-point of 1 : 640,000 for the precipitation of the product from infected plants with antisera gives us a further reason for thinking that our suspensions contain material that is not virus. The bacterial antigens that have been studied so far have, when rigorously purified, a serum precipitation end-point of 1 : 2,000,000 to 1 : 5,000,000, and these are presumably the closest analogues of the virus antigens.

The figures for four different samples of infectious material and two preparations from healthy plants are given below. The carbohydrate estimations were made by a method based on that of Tillmans and Philippi (1929):

Precipitation end-point.	Ash content (p.c.).	Carbon (p.c.).	Hydrogen (p.c.).	Nitrogen (p.c.).	Carbohydrate (p.c.).
1 : 640,000	5·0	46·0	7·3	8·8	..
	5·3	42·0	7·3	8·6	..
	5·6	49·6	6·9	9·9	12·5
1 : 320,000	..	..	..	..	10·0
Healthy product	4·0	49·6	6·6	10·7	7·3
	6·0	52·7	8·0	13·0	4·0

EFFECT OF HYDROGEN ION CONCENTRATION.

It is obvious from the methods of preparing the purified suspensions that virus " X " is precipitated by acids. The pH at which this precipitation occurs was determined by mixing purified suspensions of " X " with phthalate buffers at pHs between 3 and 6, and centrifuging off any precipitates at 16,000 r.p.m. (23,000 times gravity) for 30 minutes. The neutralized supernatant fluids and the precipitates resuspended in a little alkali were then tested separately both for their infectivity and for their power to flocculate with antiviral serum.

The supernatant fluids at pH 4 and below contained no virus, and at pH 5 and above all the virus. At pH 4·5 the virus was distributed between the supernatant fluid and the precipitate. The resuspended precipitates at pH 4 and pH 3·5 were as active as the original virus suspension, whilst at pH 3 there was some loss of activity. There was no evidence that at pHs lower than 3 the supernatant fluid could retain its activity.

Loss of infectivity produced by either acid or alkali was always accompanied by a corresponding loss in the serological reactions. The rate of inactivation of virus " X " depended both on the pH and on the temperature. At 38° C. and pH 4 half of the virus was inactivated in 24 hours and all of it in 46 hours, whereas at 0° C. and pH 4 there was no appreciable inactivation after 6 days. At pHs 3 and 10 the virus was completely inactivated after

4 hours at 38° C., and under these conditions lost three-quarters of its activity at pH 9·5.

THE EFFECT OF TRYPSIN.

Preliminary experiments indicated that potato virus " X " lost completely both the power of infecting tobacco plants and the power of reacting with antiviral serum after incubation with Merck's " pancreatin ". Merck's " pancreatin " contains about 90 p.c. lactose, and a 0·15 p.c. solution of this crude material caused a loss of 75 p.c. of the serological activity after 2 hours' incubation at pH 7 and 38° C., and complete loss of infectivity and serological reactions after 8 hours.

Pancreas extracts contain a large number of substances other than trypsin, and A. Pirie (1935) has described a factor soluble in petrol ether which inactivates certain animal viruses. We have therefore tested both petrol ether extracts of pancreas known to be free from trypsin, and trypsin known to be free from the factor for their effect on virus " X ". Both inactivated the virus, but the former was much less effective than trypsin. Furthermore, the petrol ether extracts retained their activity when boiled, whereas a boiled solution of crystalline trypsin had no effect on the virus. It is clear, therefore, that unlike the animal viruses studied by A. Pirie, virus " X " is inactivated by trypsin as well as by other constituents of pancreas.

In all the experiments to be described below a sample of crystalline trypsin* prepared by Northrop was used. Although it cannot be assumed that this contained no enzyme other than trypsin, it is certainly the purest preparation of trypsin available. Nothing which affected virus " X " could be extracted from this crystalline trypsin by petrol ether. The residue after extraction was, however, fully active.

In Table I the effects of varying both the time of incubation at 38° C. and pH 7 and the amount of crystalline trypsin mixed with suspensions of virus " X " are shown.

TABLE I.—*Effect of Crystalline Trypsin on the Reaction of Potato Virus "X".*

Suspension.	Time of incubation.	Serum dilution in optimal tube.	Infectivity: Number infec. / Number inoc.*
Virus control	3 hours	1/24	6/6
0·0250 p.c. trypsin	Tested immediately after mixing	1/24	6/6
0·0250 ,,	Kept at 0° C.	1/48	6/6
0·0002 ,,	3 hours	1/48	6/6
0·0010 ,,	,,	1/192	6/6
0·0050 ,,	,,	1/384	6/6
0·0250 ,,	30 minutes	1/192	6/6
0·0250 ,,	3 hours	No flocculation	0/10

1 c.c. of purified virus suspension mixed with 1 c.c. of trypsin solution at twice the required concentration. All incubated at 38° C. and pH 7, and diluted to 7 c.c. before testing.

* Number of plants infected. / Number of plants inoculated.

* The preparations of crystalline trypsin and pepsin were kindly provided by Dr. J. Schulman.

Eight experiments of this type were made, and in each the extent of the serological inactivation was found to vary directly with the concentration of the trypsin and with the time of incubation. The concentration of trypsin required to produce complete inactivation varied somewhat with different batches of the virus, but 0·05 p.c. acting for 3 hours at 38° C. and pH 7 was always sufficient to cause complete loss of infectivity and flocculation reactions. On occasions 0·005 p.c. acting under these conditions was found to be adequate.

It will be seen that when the virus and trypsin were mixed and tested immediately there was no effect on the serological reaction of the virus. On the other hand, the infectivity as measured by the production of local lesions was greatly reduced. The addition of an equal volume of 0·1 p.c. solution of crystalline trypsin to a virus suspension immediately before inoculation caused a fall of about 60 p.c. in the number of lesions produced, but the addition of this comparatively large amount of trypsin never caused complete loss of infectivity without incubation. It is probable that this immediate effect of trypsin is similar to that described by Stanley (1934*a*) in his work with the tobacco mosaic virus, and this will be discussed later. Apart from this immediate drop in infectivity on the addition of trypsin, the serological measurements agreed quantitatively with the number of lesions produced. Thus, the fall in the optimal flocculation point following on incubation was accompanied by a corresponding further fall in the number of lesions produced ; finally, when flocculation with antiserum was no longer obtained, the suspensions were also usually non-infectious. Occasionally, after incubation with trypsin, infections were obtained from virus suspensions which gave no reaction with antiserum. Such suspensions produced only few local lesions, and similar infections without any serological reactions were obtained with greatly diluted virus suspensions, indicating that infectivity is the more sensitive test, and detects amounts of virus too small to give any visible precipitate with serum.

A study of the effect of incubating virus " X " with crystalline trypsin at different pHs gave further evidence that the inactivation was probably due to proteolysis. Table II shows the results of one such experiment, and four others essentially similar were carried out. It is clear from this that although exposure to pH 4 alone had a slight inactivating effect, there is no evidence that trypsin had any action on the virus at a pH at which it is not proteolytically active.

We have already shown that a virus-containing precipitate is produced by bringing purified suspensions to pH 4 ; similar precipitates can be thrown down by the addition of three-fifths of a volume of saturated ammonium sulphate or by the addition of one volume of alcohol to the suspensions at about pH 5. No such precipitates are obtained with any of these reagents from suspensions which have been inactivated by incubation with trypsin at pH 7.

EFFECT OF PEPSIN.

Orientation experiments made with commercial pepsin showed that virus " X " was inactivated in acid suspensions by this mixture of enzymes. Table III gives the results obtained from an experiment repeating this, in

TABLE II.—*Effect of Crystalline Trypsin on Virus "X" at Different pHs.*

Suspensions:
1. Virus and trypsin, mixed and tested immediately.
2. Virus control at pH 4.
3. Virus and trypsin at pH 4.
4. Virus and trypsin at pH 5.
5. Virus and trypsin at pH 5·8.
6. Virus and trypsin at pH 7.

Suspension.	Time.	Dilution of antiserum. 1/12.	1/24.	1/48.	1/96.	1/192.	1/384.	0.	Infectivity: Number infec. / Number inoc.
1	13 mins.	−	+	+	−	−	−	−	6/6
	30 ,,	+	++	++	+	+	−	−	
	4 hrs.	++++	++++	++++	++++	++++	++++	−	
2	35 mins.	−	−	−	+	−	−	−	6/6
	4 hrs.	++	+++	+++	+++	+++	+++	−	
3	35 mins.	−	−	−	+	−	−	−	6/6
	4 hrs.	+	++	+++	+++	+++	+++	−	
4	16 mins.	−	+	+	−	−	−	−	6/6
	35 ,,	++	+++	+++	+++	++	+	−	
	4 hrs.	++++	++++	++++	++++	++++	++++	−	
5	12 mins.	−	+	+	−	−	−	−	6/6
	30 ,,	+	++	++	++	+	−	−	
	4 hrs.	++++	++++	++++	++++	++++	++++	−	
6	24 ,,	−	−	−	−	−	−	−	0/6

+ signs indicate the degree of flocculation.

1 c.c. of purified virus suspension mixed with 1 c.c. of 0·1 p.c. trypsin and 1 c.c. of phthalate buffer. All incubated for 3 hours at 38° C., and diluted to 7 c.c. before testing.

which Northrop's crystalline pepsin was used at a concentration of 0·02 p.c. Four other essentially similar experiments have all given similar results. This clearly shows that under these conditions virus "X" is rapidly inactivated in the presence of pepsin, and that the extent of the inactivation is proportional to the time of incubation.

TABLE III.—*Effect of Crystalline Pepsin on Potato Virus "X".*

Suspensions:
1. Virus and pepsin, mixed and tested immediately.
2. Virus control at pH 4 for 15 hrs.
3. Virus and pepsin incubated for 78 minutes.
4. Virus and pepsin incubated for 4 hrs.
5. Virus and pepsin incubated for 15 hrs.

Suspension.	Time.	Antiserum dilution. 1/16.	1/32.	1/64.	1/128.	1/256.	1/512.	0.	Infectivity: Number infec. / Number inoc.
1	25 mins.	−	−	+	−	−	−	−	6/6
	4 hrs.	++	++++	++++	++++	+++	++	−	
2	33 mins.	−	−	+	+	−	−	−	6/6
	4 hrs.	+	++	+++	+++	++	++	−	
3	55 mins.	−	−	−	−	+	−	−	4/6
	4 hrs.	−	−	−	++	++	++	−	
4	24 ,,	−	−	−	−	−	−	−	0/6
5	24 ,,	−	−	−	−	−	−	−	0/6

In each 1 c.c. of purified virus suspension was mixed with 1 c.c. 0·04 p.c. pepsin, incubated at pH 4 and 38° C., and diluted to 7 c.c. before testing.

The effect of incubating virus "X" with pepsin at different pHs gave further evidence that the inactivation was probably due to proteolysis, for, as Table IV shows, the inactivation in the presence of pepsin proceeded only at a pH at which pepsin is proteolytically active. It will be seen that although

some inactivation of the virus did occur at pH 4 in the absence of pepsin, the rate of this inactivation was small, and can be clearly differentiated from the effect of the pepsin. During the period of incubation with pepsin the precipitate produced by adjusting the virus suspensions to pH 4 disappeared.

TABLE IV.—*Effect of Crystalline Pepsin on Virus "X" at Different pHs.*

Suspensions:
1. Virus control at pH 4.
2. Virus and pepsin at pH 4.
3. Virus and pepsin at pH 4·5.
4. Virus and pepsin at pH 5.
5. Virus and pepsin at pH 5·5.
6. Virus and pepsin at pH 6.

Suspension.	Time.	Antiserum dilution. 1/16.	1/32.	1/64.	1/128.	1/256.	1/512.	0.	Infectivity: Number infec. / Number inoc.
1	16 mins.	−	−	+	−	−	−	−	6/6
	4 hrs.	++	++++	++++	++++	+++	++	−	
2	24 ,,	−	−	−	−	−	−	−	0/6
3	24 ,,	−	−	−	−	−	−	−	0/6
4	35 mins.	−	−	−	+	−	−	−	4/6
	4 hrs.	+	+	+++	+++	+++	++	−	
5	20 mins.	−	−	+	−	−	−	−	6/6
	4 hrs.	++	++++	++++	++++	+++	++	−	
6	15 mins.	−	+	+	−	−	−	−	6/6
	4 hrs.	++++	++++	++++	++++	++++	+++	−	

In each 2 c.c. purified virus suspension was mixed with 0·5 c.c. of 0·1 p.c. pepsin and 0·5 c.c. *M*/5 acetate buffer, incubated at 38° C. for 3 hours and diluted to 8 c.c. before testing.

THE EFFECT OF PAPAIN.

In the first experiments with papain equal volumes of acidified virus suspensions and a 0·2 p.c. solution of commercial papain were mixed and incubated for various times up to 5 hours at 38° C. In each sample, irrespective of the time of incubation, there developed a large precipitate and this was centrifuged off before testing. None of the supernatant fluids reacted with antiviral serum, and each gave only from 1 to 3 local lesions per inoculated leaf. The resuspended precipitates were not tested serologically, but were found to be highly infective, giving an average of 80 local lesions per inoculated leaf. It therefore seemed probable that, under these conditions, the papain acted merely by adsorbing the virus on to the precipitate. In further experiments the solutions of papain were all centrifuged at 16,000 r.p.m. before use, and by this means the formation of such precipitates was largely avoided. The usual precipitate produced by acidifying the virus suspensions of course remained, but this disappeared when the samples were neutralized before testing.

Further experiments with this centrifuged papain showed that it had no appreciable effect on either the infectivity or the serological reactions of virus "X", even after incubation periods as long as 15 hours. It is well known that papain requires activation with substances such as HCN before it can show proteolytic activity. Table V shows the results from an experiment in which 0·17 p.c. papain solution alone, 0·17 p.c. solution of neutralized KCN alone, and the two together were mixed with virus suspensions and incubated for various times at 38° C. and pH 4. Five other experiments

have all given similar results. It will be seen that inactivation of the virus was only obtained in the presence of both papain and KCN, and that under these conditions both the infectivity and power of flocculating with antiserum were lost. Further it will be seen that the loss of serological reaction in the presence of papain and KCN varied directly with the time of incubation.

At first sight it appears that the concentration of papain required to produce complete inactivation was much greater than either that of trypsin or pepsin. The papain used, however, was a crude commercial preparation of which 20 p.c. was insoluble and 75 p.c. dialysable, and its actual enzyme content could not therefore have exceeded 5 p.c.

TABLE V.—*Effect of Papain and KCN on Potato Virus " X ".*

Suspension.	Time of incubation.	Antiserum dilution in optimal tube.	Infectivity : Number infect. / Number inoc.
Virus control at pH 4	310 mins.	1/32	6/6
Virus and KCN	310 ,,	1/32	6/6
Virus and papain	150 ,,	1/32	6/6
,, ,,	310 ,,	1/32	6/6
Virus and papain and KCN	100 ,,	1/96	6/6
,, ,, ,,	150 ,,	1/256	6/6
,, ,, ,,	215 ,,	1/512	4/6
,, ,, ,,	310 ,,	No flocculation	0/6

The virus and papain suspensions were adjusted to pH 4 with *M*/50 phthalate buffer, and the KCN with HCl. Papain and KCN both used at a concentration of 0·17 p.c. All incubated at 38° C. and tested at a virus dilution of 1 in 7.

THE EFFECT OF NITROUS ACID.

The results of the enzyme experiments strongly suggest that an essential part of virus " X " is protein. If this be so, it is to be expected that the virus will contain aliphatic amino groups, and that it will be affected by substances acting on such groups. The two most easily tested substances are formaldehyde and nitrous acid. It has already been shown that formaldehyde inactivates virus " X " without affecting its flocculation with antiviral serum (Bawden, 1935). It has now been found that nitrous acid behaves similarly.

In testing the effect of nitrous acid the following method was used: Solutions of $NaNO_2$ and virus suspensions both at pH 4 were mixed and kept at 0° C. for the chosen reaction time; they were then neutralized and tested both for infectivity and for flocculating power with antiserum. Many such experiments were made, and the amount of $NaNO_2$ required to produce loss of infectivity varied considerably with different batches of the virus. The extent of the inactivation varied with both the concentration of nitrous acid and the reaction time. In one experiment complete loss of infectivity occurred with 0·36 p.c. $NaNO_2$ in 45 minutes, and with 0·18 p.c. inactivation was complete after 12 hours; 2 out of 6 plants inoculated with virus treated with 0·18 p.c. for 1 hour and 6 out of 6 plants inoculated with virus treated with

0·18 p.c. for 10 minutes became infected. With 1·4 p.c. $NaNO_2$ inactivation was complete after 10 minutes, 3 out of 6 plants inoculated with virus treated with 1·4 p.c. for 3 minutes, and all the plants inoculated with virus treated with 1·4 p.c. for a shorter time at pH 4 became infected. Suspensions exposed to 1·4 p.c. for as long as an hour reacted with antiviral serum in a manner identical with that of the untreated virus. In no instance, even with a concentration of 2·5 p.c. acid nitrite, has the reaction of the virus suspensions with antiserum been affected. It is obvious from these results that after the addition of either formaldehyde or nitrous acid to suspensions of virus " X " the close relationship that normally exists between the antigen content, as measured by the optimal flocculation method, and the virus content, as measured by the local lesion method, no longer exists. By the addition of nitrous acid to virus suspensions it was possible to obtain suspensions giving the same optimal flocculation point, but giving widely different numbers of local lesions.

The inactivating effect of $NaNO_2$ is shown only in acid suspensions. At pH 7, even 2·5 p.c. did not inactivate in 15 hours. 1·1 p.c. $NaNO_2$ completely inactivated the virus in 1 hour at pHs below 4·8, whilst suspensions kept at pH 5·2 or higher for the same time were infective. Leaves inoculated with 1 p.c. or stronger solutions of $NaNO_2$ became damaged unless washed. In all the experiments with $NaNO_2$ therefore the leaves were washed with a stream of water after inoculation. This washing did not affect the entrance of the virus, for the controls were similarly treated.

It is often difficult to determine whether the effect of a reagent is actually to inactivate the virus or merely to render the host-plant less susceptible. For the following reasons it is believed that nitrous acid affects the virus. All the suspensions inoculated to plants were at pH 7, but no loss of infectivity was found unless the suspensions had previously been made acid. Also, if the effect were on the host it might be expected to follow immediately on mixing, which it does not. Furthermore, diluting the nitrite-treated virus suspensions or removal of the nitrite by dialysis did not restore infectivity.

DISCUSSION.

Existing knowledge of the physical and chemical properties of tobacco mosaic virus and of potato virus " X " indicates that these viruses differ in many respects. Virus " X " will not withstand drying, and is much less resistant to dilution, heat, ageing *in vitro*, pH changes and chemical reagents. We have now shown that virus " X ", unlike tobacco mosaic and certain animal viruses, is rapidly inactivated in the presence of certain proteolytic enzymes. This cannot be taken as conclusive proof that virus " X " contains protein, for it is possible that even the crystalline preparations of enzymes contain substances other than trypsin or pepsin, but together with the other properties of the virus it is highly suggestive. The best evidence that virus " X " contains protein is perhaps that obtained from the experiments with papain, in which the virus was only inactivated in the presence of both papain and cyanide. The papain preparation was obviously much more likely to contain other substances than the crystalline preparations were, but its greater

usefulness is due to the fact that no other enzymes are known which are activated in this manner by cyanide.

The results of Caldwell (1933, 1935) and Stanley (1934*a*) with tobacco mosaic virus and trypsin were confirmed. A purified suspension of tobacco mosaic virus suffered an immediate loss of 60 p.c. of its infectivity as measured by the production of local lesions on *N. glutinosa* when mixed with an equal volume of 0·2 p.c. crystalline trypsin, but no further loss followed on incubation. Furthermore when diluted to 1/1000 with water the virus-trypsin mixtures were as infective as the similarly diluted virus controls. A similar immediate loss of infectivity occurred when virus " X " was mixed with trypsin, but this was always followed by complete loss of infectivity after sufficient incubation. The fact that the immediate action of trypsin does not affect the flocculation of virus " X " suspensions with antiserum, whilst on incubation the serological reactions become progressively weaker, is a further indication that the effects on mixing and on incubation are qualitatively different. It is possible that the purified suspensions of tobacco mosaic virus used by Lojkin and Vinson (1931) and Matsumoto and Somazawa (1934) were weak, and that the inactivation obtained by these workers in the presence of trypsin was due to the immediate effect of trypsin and not to proteolysis. This possibility is supported by the fact that the latter workers found that the serological reactions of their virus suspensions were unaffected even when they were non-infective. It seems, therefore, that trypsin has two distinct effects on potato virus " X ", one immediate and the other only after incubation, but that on tobacco mosaic virus the enzyme has the former effect only.

Stanley (1934*a*) has suggested that the immediate effect of trypsin is on the host-plant. Our results are not incompatible with this theory ; they would, however, fit other theories. For example, trypsin may form a loose complex with the virus, which leaves the serological reactions unaffected and which breaks down on dilution. Stanley tried to disprove this possibility by measuring the rate of diffusion of trypsin in the presence and absence of tobacco mosaic virus. He found no differences, and took this as evidence that no such complex was formed. In view of the lack of knowledge on the possible combining ratios of trypsin and tobacco mosaic virus and on the number of virus particles in the suspension used, it is quite possible that such an excess of trypsin was used that no differences in the rates of diffusion could have been measured even if such a complex were formed.

Stanley (1934*a*) further states that globin had an effect similar to that of trypsin in reducing the infectivity of tobacco mosaic virus, but it is noticed that in the experiments quoted by him globin was used at 8 times the concentration of commercial trypsin and other proteins tested. With suspensions of virus " X " the addition of an equal volume of 0·2 p.c. globin had no effect on the number of local lesions produced, whereas the addition of an equal volume of 0·2 p.c. crystalline trypsin to a sample of the same batch of virus reduced the number of lesions from 567 to 171. The inoculations were made so that the control suspension was inoculated to one half and the virus-trypsin mixture to the opposite half of the same leaves.

In the presence of pepsin, as in the presence of trypsin, tobacco mosaic virus and potato virus " X " behave differently. Various workers reported

that pepsin had no effect on tobacco mosaic virus, whilst Stanley (1934*b*) found that crystalline pepsin slowly inactivated it, complete inactivation being obtained only after several days' incubation at pH 3 and 37° C. in the presence of 0·17 p.c. pepsin. Virus " X ", on the other hand, was completely inactivated by a 0·02 p.c. solution of crystalline pepsin in 3 hours at pH 4 and 38° C.

SUMMARY.

The experiments recorded were made with purified suspensions of the S strain of potato virus " X ". The method of preparing the suspensions and analyses of the material in them are given. A factor in pancreas which is soluble in petrol ether inactivated the virus. The addition of trypsin immediately reduced the infectivity without affecting the flocculating power with antiserum. Incubation with crystalline preparations of trypsin and pepsin destroyed both the infectivity and the power of reacting with antiserum. The inactivation occurred only at pHs at which the enzymes are proteolytically active, and the extent was directly proportional to the concentration of the enzyme and to the time of incubation. Papain alone and cyanide alone had no effect on the virus, but the two together inactivated it. Nitrous acid caused loss of infectivity without affecting the reactions with antiserum. The extent of the loss was proportional to the concentration of nitrous acid and to the reaction time.

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THE CARBONIC ANHYDRASE CONTENT OF BLOOD IN PATHOLOGICAL STATES IN MAN.

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THE work of Meldrum and Roughton (1933) on the occurrence and properties of carbonic anhydrase in mammalian blood has prompted an investigation into the carbonic anhydrase content of blood in pathological states in man. The chemical reactions which lead to CO_2 evolution in the lung from the bicarbonate in the blood are as follows :

$$HP \text{ (protein acid)} + NaHCO_3 \rightarrow NaP \text{ (protein salt)} + H_2CO_3 \quad (1)$$

$$H_2CO_3 \rightarrow CO_2 + H_2O \quad (2)$$

Of these reactions the first, being purely ionic, is very rapid, but the second is slow and must thus limit the rate of CO_2 escape into the expired air. Using the velocity constants of previous workers, Henriques (1928), calculated that the rate at which CO_2 could be formed by the second reaction, under physiological conditions, would be far less than that actually observed in the expired air. When he compared the rate at which CO_2 is evolved when hæmoglobin solutions, on the one hand, and serum solutions, on the other hand, were violently shaken *in vacuo*, he noted that CO_2 came off much more quickly from the hæmoglobin solution. Although Henriques did not interpret these results on the basis of a catalyst mechanism, Hawkins and van Slyke (1930), in experiments of a similar nature, found distinct evidence of a catalyst, and Brinkman and Margaria (1931) showed that blood or hæmoglobin, even when diluted 1 part in 20,000, accelerated the evolution of CO_2 from mixtures of bicarbonate with buffers of physiological pH. Finally Meldrum and Roughton (1932) isolated from ox blood a substance of which 1 part in 10,000,000 accelerated the reaction, and this they called carbonic anhydrase.

Meldrum and Roughton (1933) gave methods for its preparation and carried out investigations as to its properties and distribution. The purest preparation is free from hæmoglobin, hæmatin, catalase, peroxidase and oxidase. The enzyme is very stable in the solid condition, and is also stable in solution over the range pH 3–12. Its solution in water is destroyed by 30 minutes heating at 65° C. ; it shows most of the protein reactions, and it accelerates a number of processes in which the over-all velocity depends upon the rate of one or other of the reactions $CO_2 + H_2O \rightleftharpoons H_2CO_3$ or $CO_2 + OH^- \rightleftharpoons HCO_3^-$. The enzyme is poisoned by carbon monoxide, cyanides, sulphides, azides, copper, silver, zinc, mercury and phenylurethane. The amount of carbonic anhydrase in mammalian blood was found to vary widely not only in various species, but in members of the same species.

Inasmuch as carbonic anhydrase is a substance of prime physiological

significance—without it CO_2 could not be excreted nearly fast enough for the needs of the body—it is clearly of importance to follow its distribution in pathological conditions, particularly those associated with marked changes in the blood and those with disturbances of respiration. The present work, of which the results are given below, has been designed to investigate this aspect of the subject.

METHODS.

The method adopted was to measure the catalytic effect of the enzyme on the rate of evolution of CO_2 from sodium bicarbonate solutions when mixed with phosphate buffer of pH 6·8. This was carried out with the aid of the boat apparatus of Brinkman, Margaria and Roughton (1933), as used by them in their work on the kinetics of the reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ in the absence of a catalyst. A glass boat with a ridge up the middle separating two compartments was used as the receptacle for the solutions. In one compartment was placed 2 c.c. of the phosphate solution and in the other 2 c.c. of the sodium bicarbonate solution. The boat was placed in a water-bath kept at 15° C., time for temperature equilibration was allowed, and it was then shaken rapidly by a mechanical shaking device electrically driven. It is of importance that the speed of shaking be rapid and constant, otherwise the rate of evolution of CO_2 is a function of the speed of the motor ; therefore the speed of shaking is adjusted by a variable resistance until an increase in rate causes no increase in the rate of evolution of the gas.

The evolution of CO_2 was followed by noting the manometer reading at 0, 5, 10, 15, 30, 45, 60, 90 and 120 seconds. This observation of the rate is a true measure of the velocity of the chemical reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$, diffusion from liquid to gas phase not being a limiting factor provided that the first half of the total CO_2 is evolved in not less than about 15 seconds.

The phosphate solution used by Meldrum and Roughton was made by mixing equal volumes of $M/5$ Na_2HPO_4 and $M/5$ KH_2PO_4, and the bicarbonate solution consisted of $M/5$ $NaHCO_3$ dissolved in $M/50$ NaOH. During the course of this work it was found that their bicarbonate solution did not remain entirely stable, but that the volume of CO_2 evolved fell appreciably after standing. Ultimately, however, this volume reached a steady value. This solution was then titrated with $M/10$ HCl, the pH change being followed electrically, and a titration curve constructed. From this curve the titrations corresponding to the pH values 8·2 and 3·6 were read off, and these indicated that the solution had the composition $0{\cdot}038M$ in NaOH and $0{\cdot}186M$ in $NaHCO_3$. A solution of this composition was then made up, and was found to be stable.

Having done a control experiment as above described, readings were obtained in which 1/10 c.c. of suitably diluted blood was added either to the phosphate or the bicarbonate, and the amount of enzyme present calculated.

Calculation of the amount of enzyme.—Curves were constructed for both the catalysed and uncatalysed reaction in which the pressure of CO_2 in mm. of H_2O was plotted against the time in seconds. The average rate of CO_2 evolution during the second quarter of the process was given by the reciprocal of the time taken. If then R_0 be the rate in the absence of the catalyst and R the rate in

the presence of catalyst the fraction $\frac{R-R_0}{R_0}$ is a suitable measure of the catalytic activity. It is necessary to assign a value to the amount of enzyme present, and one unit is arbitrarily defined as that amount of enzyme which, when dissolved in 4 c.c. of the phosphate-bicarbonate mixture, gives a value of $\frac{R-R_0}{R_0}$ equal to 1 at 15° C. If the temperature differs slightly from 15° C. a correction can be made by applying a temperature coefficient of 3·6 per 10° C. for the uncatalysed reaction and of 2·1 for the catalysed reaction. The most accurate results are obtained when the amount of catalytic material added is such as to increase the rate of the process about three or four times, since the relation between enzyme concentration and rate of reaction is still linear up to this point. Knowing the dilution of the blood that has been used in any experiment, the amount of carbonic anhydrase can be expressed as units per 10 cmm. of whole blood.

RESULTS.

The occurrence of carbonic anhydrase in the blood of normal individuals was first investigated. The subjects chosen were a group of medical students. The results are given in Table I. In each case the percentage of red cells in the blood was obtained by the hæmatocrit. The figure obtained by dividing the anhydrase value by the hæmatocrit reading will be designated the carbonic anhydrase index in the following table:

TABLE I.

Subject No.	Units carbonic anhydrase per 10 cmm. blood.	Hæmatocrit reading p.c. cells.	Carbonic anhydrase index.
1	16·5	46	0·36
2	15·5	40	0·39
3	14·4	44	0·33
4	15·0	40	0·37
5	14·0	44	0·32
6	17·5	45	0·39
7	15·1	47	0·32
8	14·6	42	0·35
9	16·5	43	0·38
10	13·9	46	0·30

It will be seen that in this series there is no close relationship between the amount of carbonic anhydrase present and the percentage of red cells; the range of variation in the index values is as great as that in the carbonic anhydrase. No significant diurnal or day-to-day variation in the carbonic anhydrase content of any individual subject was observed.

The results obtained from an examination of the blood in pathological states is given in Table II.

TABLE II.

Case No.	Diagnosis.	Units carbonic anhydrase per 10 cmm. blood.	Hæmtaocrit reading p.c. cells.	Carbonic anhydrase index.
1	Secondary anæmia	4·7	..	..
2	,, ,, emphysema	6·6	16	0·41
3	Secondary anæmia	9·5	25	0·38
4	,, ,, cancer of stomach	6·0	12	0·50
5	Secondary anæmia, uræmia	6·0	12	0·50
6	Pernicious anæmia	6·1	12	0·51
7	,, ,,	20·7	21	0·99
8	Auricular fibrillation, cyanosed	18·2	48	0·38
9	Congestive heart failure	27·0	52	0·52
10	Bronchitis, cyanosed	17·2	45	0·38
11	Cardiac failure	15·2	50	0·30
12	"Cushing's syndrome," cyanosed from acetanilid	18·5	40	0·46
13	Polycythæmia vera	21·6	60	0·36
14	Jaundice	21·6	40	0·54
15	,,	24·3	43	0·57
16	,,	17·6	37	0·48
17	Essential hypertension	15·0	..	..
18	,, ,,	15·2	38	0·40
19	,, ,,	21·7	47	0·46
20	,, ,,	13·5	46	0·30
21	Hyperthyroidism	14·7	37	0·40
22	,,	14·6	39	0·37
23	,,	17·0	46	0·37
24	Renal colic	16·5	..	..
25	Diabetes mellitus	13·0	..	..
26	Asthma	12·0	..	..
27	Acromegaly	13·4	30	0·44
28	Lymphatic leukæmia	13·8	39	0·35
29	Cancer of breast	14·2	35	0·40
30	Duodenal ulcer	17·2	34	0·50
31	Alkalosis	12·2	31	0·40
32	Pneumonia	17·2	47	0·37
33	Mitral stenosis	14·3	47	0·30

DISCUSSION.

In this series of pathological cases the amount of carbonic anhydrase varies over a wide range from 4·7 to 27·0 units per 10 cmm. blood as compared with a range of 13·9 to 17·5 units for normal subjects. The hæmatocrit values

for the two groups range from 12 to 60 p.c. of cells and 40 to 47 p.c. of cells respectively. Since cases are encountered that have a very low absolute carbonic anhydrase content in the blood and yet do not show marked impairment of respiratory function, it is reasonable to infer that in normal individuals there must be a considerable excess of enzyme over ordinary requirements. The data recorded in Tables I and II as regards units of carbonic anhydrase and readings of the hæmatocrit are plotted in Fig. 1, which in the main shows a rough correspondence between the two.

The most obvious condition in which to find an abnormal anhydrase content would be anæmia. Low absolute values for carbonic anhydrase are shown in

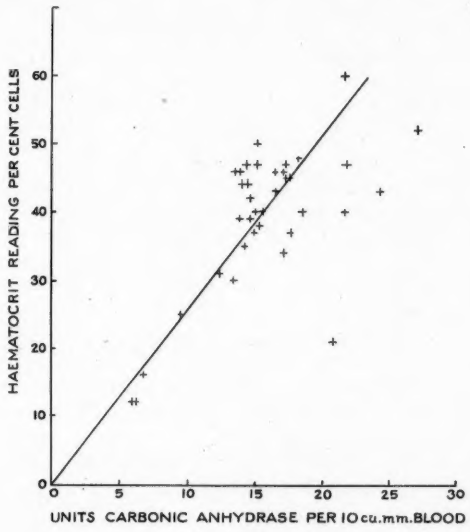

FIG. 1.

all cases except one—a case of pernicious anæmia, No. 7 in the series. This case had 20·7 units of anhydrase, an icteric index of 21, and a reticulocyte count of 19 p.c. Case No. 6, also a case of pernicious anæmia, showed 6·1 units of anhydrase, but only an occasional reticulocyte was present. The high anhydrase value of Case No. 7 may thus possibly be related to the number of reticulocytes present in the blood. All the other cases of anæmia show low anhydrase values, and thus there is a rough correspondence between the low anhydrase content and the low hæmatocrit reading. However, the anhydrase index is on the average higher than for the normals, and the inference is that the blood-corpuscles, less in number, are carrying more anhydrase.

Cases Nos. 8, 9, 10, 11, 12, of cyanosis show an increased amount of carbonic anhydrase in the blood. All these cases show an increase in the hæmatocrit reading except Case No. 12—a case of cyanosis from acetanilid poisoning,

which gave a positive test for methæmoglobin in the blood. Case No. 13, a case of polycythæmia vera, although having the highest hæmatocrit reading, has an anhydrase content only proportionately high, thus giving an anhydrase index within the normal range.

All the cases of jaundice have raised anhydrase values, and at the same time normal hæmatocrit readings, with the result that the anhydrase indices are raised above the normal in every case. It is possible that this finding may be due to the lowering of surface tension due to the presence of bile-salts. If *in vivo* the enzyme is within the corpuscle, the substrate must diffuse inside the cell in order that the reaction may take place; if, on the other hand, the enzyme is located on the surface of the corpuscle, a decrease of surface tension might facilitate the reaction between enzyme and substrate. This increase in carbonic anhydrase in jaundice would thus seem to favour the supposition that the enzyme is situated at the surface of the corpuscle.

SUMMARY.

The distribution of carbonic anhydrase has been followed in the blood of normal subjects and in patients with various pathological conditions.

The absolute amount of carbonic anhydrase is decreased in anæmia and increased in cyanosis. There is usually a rough correspondence between the amount of carbonic anhydrase in the blood and the percentage of corpuscles as given by the hæmatocrit. Exceptions to this relation were observed, notably in cases of jaundice.

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THE Vi ANTIGENS OF VARIOUS SALMONELLA TYPES.

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SINCE the presence in *S. typhi* of the so-called Vi antigen was first established (Felix and Pitt, 1934*a* and *b*), various attempts have been made to demonstrate antigens of this type in other members of the salmonella group. These earlier attempts have failed for the following reasons: In *S. typhi* the two extreme types of strain, namely, the one inagglutinable and the other highly agglutinable by the O antibody, are not uncommon, and the mere existence of the two strikingly different types called for a study of the phenomenon, and in addition served as a useful guide to the presence of the Vi antigen. In the other salmonella species, however, which we have tested up to the present, *i. e. S. paratyphi* A and B, *S. aertrycke* and *S. enteritidis* Gaertner, similar types of high agglutinability or extreme inagglutinability by the O antibody have not yet been found. The problem of demonstrating *in vitro* a hypothetical Vi antigen in these organisms could, therefore, be approached only by using immune sera from which the O antibody had been removed completely.

Now from experiments with *S. typhi* it was known that its Vi antigen (though in some respects even more labile than the H antigen) was still capable of specifically absorbing Vi antibodies even after the suspensions had been heated at 100° C. for one hour, though their absorbing power was much lower than that of unheated suspensions (Felix, Bhatnagar and Pitt, 1934). The preparation of a "pure" Vi serum by means of absorption with a steamed suspension of a Vi-containing strain is, therefore, not a satisfactory method, since the low-titre Vi antibody may be entirely removed or considerably reduced before complete absorption of the high-titre O antibody has been accomplished.

A reliable method for separating Vi from O antibodies was found by the use of acid-treated bacteria. It is known from earlier work that O antigens are highly resistant to the action of hydrochloric acid even in relatively strong concentrations (Weil and Felix, 1920). It has now been found that suspensions of Vi-containing strains of *S. typhi* after treatment with 5 p.c. HCl at room temperature no longer absorb the Vi antibody, whereas their power of absorbing O antibodies remains unimpaired. On the other hand, suspensions treated with alcohol still absorb both the Vi and the O antibodies, though the agglutinability by the Vi antibody of alcohol-treated suspensions is almost annulled,

or at least very much reduced. The agglutinogenic properties of both the Vi and the O antigens of *S. typhi* also are not impaired by treatment with alcohol.

When sera from rabbits immunized with living bacilli of the various salmonella types were absorbed with acid-treated suspensions of the homologous strains, no difficulty was experienced in demonstrating the presence in the sera of antibodies corresponding to the Vi antibody of *S. typhi*. The general lines on which these tests were conducted are illustrated in Table I.

TABLE I.—*The Demonstration of Vi Antibody in Sera from Rabbits Immunized with Living Bacilli of Various Salmonella Types.*

Agglutination of suspensions of the homologous strain.	Immune serum absorbed with the homologous strain.			
	Live.	Treated with—		Heated 2½ hours at 100° C.
		Alcohol.	HCl.	
Heated 2½ hours at 100° C..	—	—	—	—
Treated with HCl . .	—	—	—	—
Treated with alcohol . .	—	—	+	+
Live	—	—	+	+

NOTES.—*Preparation of acid-treated suspensions.*—Cultures grown in Roux bottles on plain agar for 24 hours at 37° C. were suspended in 20 c.c. of saline and an equal volume of 10 p.c. HCl was added. The growth from one Roux bottle of the usual size (8 1/2 in. by 4 1/2 in.) yielded from 1,600,000 to 2,400,000 million organisms. The suspensions were kept in flasks containing glass beads and were occasionally shaken. After two days at room temperature they were centrifuged, the supernatants removed, and the sediments resuspended in 40 c.c. of saline and neutralized by the addition of dilute NaOH. Finally the sediments were washed four times on the centrifuge, each time with 40 c.c. of fresh saline.

Preparation of alcohol-treated suspensions.—The growth from one Roux bottle was washed off with 10 c.c. of saline into flasks containing glass beads and 30 c.c. of 96 p.c. alcohol were added. After two days at room temperature (in the dark) the suspensions were centrifuged and washed four times on the centrifuge with fresh saline.

Table I shows that the absorption with steamed or acid-treated bacilli removed all the agglutinins capable of reacting with steamed or acid-treated organisms, but still left some agglutinins which caused the live and the alcohol-treated bacilli to clump. This clumping occurred in the typical granular fashion known from O and Vi agglutination. The relative sensitiveness to O agglutinins of the four different suspensions employed was, of course, carefully checked by controls with a pure O serum of known titre, and there was no doubt whatsoever that we were dealing with an antibody separate and distinct from the O and H antibodies. For the sake of simplicity the H agglutinins which also were present in the sera are not recorded in this and the following tables. The floccular H agglutination does not cause any real source of error in the correct reading of the granular Vi agglutination.

The details of these absorption tests, which were conducted according to

the rules applied to strictly quantitative tests, need not be described here. With *S. paratyphi* A and B and with *S. aertrycke* the results were perfectly clear-cut and uniform, provided the quantity of absorbing bacilli was not grossly excessive. In the latter case Vi agglutinins may not be demonstrable. It is not yet clear whether this is due to specific absorption by some residual Vi antigen, still present in active form in the acid-treated suspensions, or to the well-known non-specific effect of the concentrated extract derived from the enormous number of bacillary bodies that are brought into contact with a relatively small quantity of serum. The titres of Vi agglutination observed with the various salmonella sera were of the same order as those previously described in the case of immune sera to *S. typhi*. The maximum titres recorded with some of the salmonella sera were 1 in 1000 or 1 in 2000, when estimated against those strains that showed the highest agglutinability by Vi antibody. The O titres were on the average ten times higher.

So far we have not succeeded in demonstrating Vi antibodies in the serum of rabbits immunized with *S. enteritidis* Gaertner but only a few strains have so far been tested. In the case of *S. paratyphi* A and B and *S. aertrycke*, however, absorbed Vi antisera, which were entirely devoid of O antibodies, were available for cross-agglutination tests with the different salmonella types under investigation. In some instances cross-absorption tests were also carried out. The results are summarized in Table II.

TABLE II.—*Cross-Agglutination Tests with Vi Sera.*

Agglutination of living organisms.	Vi antisera absorbed with acid-treated homologous bacilli (entirely devoid of O agglutinins).			
	T.	A.	B.	Aertrycke.
T	+	−	−	−
A	−	+	−	−
B	−	−	+	+
Aertrycke	−	−	+	+
C	(±)	−	−	−
Gaertner	−	−	−	−

Again the floccular H agglutination occurring with some of these sera is disregarded, and the granular Vi agglutination only is recorded in Table II. There is no overlapping in the Vi antigens of *S. typhi* and the other species, with the exception of *S. paratyphi* C, already described by Kauffmann (1935). The Vi antigen of *S. paratyphi* A so far appears to be strictly specific. On the other hand, the Vi antigen of *S. paratyphi* B seems to be identical with that of *S. aertrycke*. What exactly is the degree of overlapping between *S. paratyphi* C and *S. typhi* has not yet been investigated, and this is indicated in the table by a ± in brackets. *S. enteritidis* Gaertner does not seem to share any of the three different Vi antigens.

The scheme reproduced in Table II is, of course, quite provisional, since it is

based on the examination of only a few strains of each species. It will grow more complicated as the other recognized salmonella types are included.

Now the question arises, on what grounds are these salmonella antigens classified as Vi antigens, together with that known in *S. typhi*, where the relationship to virulence has been well established? So far evidence of a similar relationship between the specific Vi antigen and the virulence of the organisms has been obtained in the case of *S. paratyphi* A and B. We do not yet commit ourselves with regard to *S. aertrycke*, though with this species, too, some preliminary tests seem to point in the same direction. Some strains of *S. aertrycke* were found to contain Vi antigen, others to be devoid of it.

When a number of strains of *S. paratyphi* A or B are tested against pure O antisera the difference in agglutination titres may be twofold or threefold, whereas with *S. typhi* the difference may be as great as fifty- or a hundredfold, or even more. In Vi agglutination, on the other hand, A and B strains behave in the same manner as *S. typhi*. The more Vi antigen present in a strain the lower is its Vi agglutinability and *vice versa*. This simple test, however, is only of limited value for the quantitative estimation of Vi antigen, since the rule is valid only so long as the quantity of Vi antigen present in a strain does not fall below a certain minimum. The most reliable method for estimating the content of Vi antigen is absorption of a Vi serum with suspensions of living organisms. By this procedure it can be shown that the more Vi antigen present in a strain of *S. paratyphi* A or B, the higher is its virulence. This is illustrated in Table III.

TABLE III.—*Virulence of Strains of* S. paratyphi *B in Relation to their Content of Vi Antigen.*

Absorption of Vi Antibody.

	Serum dilutions.	Vi serum made with live organisms of strain 18142 and absorbed with acid-treated organisms of strain 18142.				
		Control not further absorbed.	Further absorbed with living organisms of strains.			
			158.	359.	3.	18142.
Agglutination of living organisms of strain 18142	1 : 100	+++	+++	+	+	+++
	1 : 200	+++	++	−	−	++
	1 : 500	+++	+	−	−	+
	1 : 1000	+	−	−	−	−
	1 : 2000	−	−	−	−	−

Virulence to Mice.

		Organisms of strains.			
		158.	359.	3.	18142.
Test dose intraperitoneally	200 × 10^6	1/4	4/4	4/4	0/4
	100 × 10^6	0/6	6/6	6/6	0/6

NOTE.—The numerators of the fractions denote the number of animals that died, the denominators the number inoculated.

Four strains of *S. paratyphi* B are compared in Table III with regard to their virulence for mice and their capacity to absorb Vi antibody. It is seen that the two strains of low absorbing power are of low virulence, whereas the two strains of high absorbing power are of high virulence. Thus the analogy to the conditions obtaining in *S. typhi* is quite evident.

Further analogies were found in experiments on passive immunization of mice against infection with *S. paratyphi* B, and in tests demonstrating the neutralization of the toxic effect of this organism. The technique of these protection tests was that described in previous papers (Felix and Pitt, 1934*a* and *b*). The results may be summarized as follows:

Pure O sera as well as pure Vi sera protected mice against infection with the most virulent strain of *S. paratyphi* B at our disposal. The potency of the Vi antibody acting alone was at least as great as, if not greater than that of the O antibody in protective action. Thus the difference in the protective value of the two types of antibody is much less striking here than it is with the most virulent strains of *S. typhi*. This is exactly what was to be expected, since in the various salmonella types the presence of the Vi antigen does not cause inhibition of the interaction between the O antigen and its antibody in the same degree as it does in the most virulent strains of *S. typhi*. In this respect the common salmonella strains may well be compared with the most common type of *S. typhi*, namely the type of intermediate virulence and agglutinability.

The tests for the neutralization of the toxic effect on mice of vaccines of *S. paratyphi* B, killed by heating at 58° C., gave the same results as those previously described with *S. typhi*. Of the three different antibodies, H, O and Vi, present in the rabbit immune sera, it was the O antibody alone that showed a definite relationship to the endotoxin-neutralizing power of the serum.

In both these types of protection tests, which so far were carried out only with *S. paratyphi* B, immune sera against *S. aertrycke* strains were used as well as homologous sera. No difference in the Vi or O antigens of the two organisms was discernible by this method. On the other hand, the striking effect on virulence of growing cultures of *S. typhi* at various temperatures (Felix, Bhatnagar and Pitt, 1934) could so far not be demonstrated with the other salmonella types. Cultures of A and B and aertrycke strains, grown at 21° C. for more than two weeks, were found to be as virulent and as rich in Vi antigen as those grown at 37° C. *S. typhi* also differs from the other salmonella species with regard to the agglutinability by the Vi antibody of suspensions treated with alcohol. The Vi agglutinability of suspensions of *S. typhi* treated with 75 p.c. alcohol is very much reduced, whereas that of suspensions of *S. paratyphi* A and B and *S. aertrycke* is not impaired. High-titre Vi antisera can, however, be obtained by immunizing rabbits with alcohol-treated suspensions of *S. typhi*. Should further investigation show that the Vi antibody elaborated in response to immunization with alcohol-treated suspensions is not deficient in protective power (Felix and Bhatnagar, 1935), the use of such suspensions may prove to be of practical value in the preparation of vaccines and therapeutic sera.

CONCLUSION.

It is concluded from these experiments that antigens of the type of the Vi antigen of *S. typhi* also occur in other salmonella species, and there, too, play an important *rôle* in infection and immunity.

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